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(54) Title: IDENTIFICATION OF GLYCOSPHINGOLIPIDS THAT PROMOTE HIV-1 ENTRY INTO CELLS

(57) Abstract

The invention is related to the discovery of cofactors that promote CD4–dependent HIV–1 fusion and entry. More specifically, disclosed herein are biotechnological tools, diagnostics, prophylactics, therapeutics and methods of use of the foregoing for the treatment and prevention of HIV–1 infection that exploit interactions between gp120–gp41 and glycosphingolipids.

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IDENTIFICATION OF GLYCOSPHINGOLIPIDS THAT PROMOTE HIV-1 ENTRY INTO CELLS

FIELD OF THE INVENTION

The present invention is related to the discovery of cofactors that promote CD4-dependent HIV-1 fusion and entry. More specifically, disclosed herein are biotechnological tools, diagnostics, prophylactics, therapeutics and methods of use of the foregoing for the treatment and prevention of HIV-1 infection that exploit interactions between gp120-gp41 and glycosphingolipids.

BACKGROUND OF THE INVENTION

Human immunodeficiency virus type 1 (HIV-1) enters permissive cells by binding to the cellular receptor, CD4, followed by gp120-gp41 mediated fusion of the viral and target cell membranes. (Maddon et al. *Cell* 47:33 (1986)). In addition to CD4, several members of the chemokine-receptor family have been shown to act as necessary coreceptors for HIV-1 fusion and infection. Chemokine-receptor usage varies depending on the viral strain and the target cells used and is the primary determinant of viral tropism. Although the discovery of chemokine receptors has revolutionized our thinking about the way HIV-1, HIV-2, and the simian immunodeficiency virus gain entry into the cell, some crucial pieces of the puzzle are missing.

BRIEF SUMMARY OF THE INVENTION

The present invention discloses biological tools, diagnostics, prophylactics, therapeutics and methods of use of the foregoing for the treatment and prevention of HIV-1 infection and/or AIDS. In an embodiment that has many biotechnological and clinical applications, fusion of a cell with a virus, a second cell or a liposome is enhanced by providing a glycosphingolipid (GSL). Preferably, fusion is enhanced and executed, according to this embodiment, by providing globotriaosylceramide (Gb3) to a CD4 expressing cell such that Gb3 is incorporated in the cell's plasma membrane and the cell is brought into contact with a virus, a second cell or a liposome that has on its surface gp120-gp41. Cells and liposomes whose concentration of cell surface GSLs (e.g., Gb3) has been manipulated by liposome mediated-transfer or FACS-based selection methods, for example, are further aspects of this embodiment that are useful as HIV therapeutics, drug delivery agents and biotechnological tools. Advantageously, HIV and T-cells expressing gp120-gp41 can be selectively targeted by the enhanced cells or liposomes of the invention or both so that novel HIV treatment protocols can be developed.

Other embodiments concern a variety of supports joined to a GSL, preferably Gb3 or Gb3 carbohydrate head groups, which can also have attached CD4 or a chemokine receptor. These embodiments have many biotechnological, diagnostic and therapeutic uses including the isolation of HIV, the purification of HIV contaminated solutions, the rapid detection of HIV in fluids from infected individuals and the removal of HIV in humans by a portable dialysis system. In one aspect of this embodiment, for example, multimers of natural or synthetic Gb3 are immobilized to a macromolecular structure, such as plastic, polystyrene, polyethylene, polyvinyl, protein, cellulose, starch, glycogen,

chitosane, aminated sepharose, silica gel, zeolite, diatomaceous earth or aminated glass, and the GSL-support is used to isolate HIV by affinity chromatography or to remove HIV from a contaminated solution, such as a human fluid. Similarly, the GSL-supports, as described herein, can be incorporated into diagnostic tools for the rapid detection of HIV and can also be incorporated into a novel dialysis system that can purify the blood of an HIV infected patient in the privacy of their own home.

Agents for the treatment and prevention of HIV infection and/or AIDS further embody the invention. In one aspect, agents that inhibit the synthesis of glycosphingolipids (GSLs), glucosyl ceramide or Gb3 are administered to a subjected suffering from HIV and/or AIDS. The agent can be an amino-sugar derivative such as N-butyl or N-hexyldeoxygalactonojirimycin or 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol or analogs thereof, for example. In another aspect of this embodiment, ribozymes or antisense oligonucleotides complementary to enzymes involved in the biosynthesis of GSLs, glucosyl ceramide or Gb3 are used. Further, the use of monoclonal antibodies to inhibit GSL, glucosyl ceramide or Gb3 synthesis or transport is disclosed in the invention.

In another aspect, agents that interfere with the binding of GSLs, glucosyl ceramide or Gb3 are administered so as to treat and prevent HIV infection and/or AIDS. Agents such as glycosphingolipidases, glucosyl ceramidases or globotiaosylceramidases (enzymes that cleave sugar residues on GSLs, glucosyl ceramide or Gb3, respectively), monoclonal antibodies, lectins, chemicals, recombinant proteins, toxins and synthetic peptides are disclosed as prophylactics or therapeutics that prevent or treat HIV infection and/or AIDS. Specifically, a verotoxin, mutant verotoxin or heteromultimer of verotoxin subunits can be used for the prevention and treatment of HIV infection and/or AIDS.

Additionally, GSLs, glucosyl ceramide, Gb3 or Gb3 carbohydrate head groups can be administered to a patient in need so as to prevent or treat HIV infection by competitive inhibition. The GSLs, glucosyl ceramide, Gb3 or Gb3 carbohydrate head groups can be provided in a natural or synthetic form and can also be multimeric. Additionally, the GSLs, glucosyl ceramide, Gb3 or Gb3 carbohydrate head groups can be given in a form that is coupled to a macromolecular support. This later formulation has the added advantage of clearing HIV from the body.

The agents for the treatment and prevention of HIV infection and/or AIDS mentioned above can also be administered in conjunction with an agent that modulates the activity of gp170 so as to overcome the multi-drug resistant phenotype. Further, the methods of the invention include the treatment or prevention of HIV infection and/or AIDS by administering an agent that selectively modulates the activity of gp170.

In another embodiment, methods of screening for prophylactic or therapeutic agents or fusion enhancing agents are provided. One approach disclosed herein, for example, involves the immobilization of either Gb3 or gp120-gp41 to a support followed by the addition of a complementary molecule (e.g., Gb3 complements gp120-gp41 and vice versa). Prophylactic agents can then be screened by contacting the immobilized Gb3 or gp120-gp41 support with the candidate agent prior to the addition of the complementary molecule. Additionally, therapeutic agents can also be screened for their ability to out compete or displace binding of the complementary molecule by providing the agent

either simultaneously or after providing the complementary molecule. Enhancing agents can also be identified in a similar manner by providing the agent either before, during or after the addition of the complementary molecule.

In another aspect of this embodiment, methods of screening for prophylactic or therapeutic agents or fusion enhancing agents using a cell fusion-based assay are described. By this approach a cell having CD4 is treated with a potential prophylactic, therapeutic or fusion enhancing agent, as above, and the ability of the CD4 cell to fuse with a liposome, a virus or a second cell having gp120-gp41 is determined.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. This figure shows the effect of GSL depletion on HIV-1 infection. HIV-1 particles containing the NL4-3r'e genome and having gp120-gp41 derived from different isolates were prepared as described in EXAMPLE 1. GHOST (3) parent (expressing CD4 only), and GHOST-X4, -R5, and -345 cells were grown in medium containing 10 M PPMP for at least 7 days prior to the assay. Cells were plated in 12-well plates (1.5×10^4 cells/well) and medium containing viral particles added. Four days after infection, the cells were examined microscopically for GFP expression as described in EXAMPLE 1. % Infectivity = 100 x [(number of clusters of GFP-positive cells x average number of cells per GFP-positive cluster) / total number of cells observed]. Three separate experiments yielded the same results.

FIGURE 2. This figure shows the results of thin layer chromatography analysis of GSL levels in control and PPMP-treated cells. GSLs were isolated as described in EXAMPLE 1 from Ghost 345 cells that were either untreated, or treated for at least seven days in culture medium containing 10 M PPMP. Monosialoganglioside standard (10 g), containing GM3, GM2 and GM1, and lipid extracts from $5x10^6$ cells were spotted onto a 20 x 20-cm silica-gel TLC plate (Fisher, Malvern, PA), which was developed in $CHCl_3$/MeOH/10%$KCl_{(aq)}$ (50:40:10, vol/vol). After running, the plate was air dried, sprayed with resorcinol, and heated to develop the spots. Images were taken on a BioRad Fluor-S Multilmager, using white light epi-illumination. Bands, indicative of GSLs interacting with resorcinol, are outlined in the standard and untreated lanes. Notice the down-regulation of GSLs in PPMP-treated cells compared to control cells (see arrows).

FIGURES 3A, B, and C These figures show the results of the flow cytometry analysis of CD4 and chemokine receptor expression in control and PPMP-treated cells. Expression levels of CD4, CXCR4 and CCR5 were examined on control (left panel) and PPMP treated cells (right panel). Cells were incubated for 1 hr at 4 °C with either (A) PE-conjugated mouse IgG anti-CXCR4 (12G5), (B) PE-conjugated mouse IgG anti-CD4 (OKT4), or (C) mouse IgG anti-CCR5 (2D7), followed by FITC-labeled goat IgG anti-mouse antibody. Fluorescence was examined with a Becton Dickinson FACScalibur at 10,000 events/sample. Unlabeled cells and cells incubated with FITC anti-mouse, but not anti-CCR5 antibody were used as negative controls.

FIGURE 4This figure shows the migration of GHOSTX4 cells induced by SDF-1 .

Different concentrations of SDF-1 were placed in the lower wells of the chemotaxis chamber; cells were placed in the upper wells, which were separated from the lower wells by a ploycarbonate filter. The results are expressed as a chemotaxis index (CI) representing the fold increase of migrating cells in response to SDF1- over the response to control medium. Significant cell migration ($p < 0.05$) was detected with 10 ng/ml SDF1- present as chemoattractant.

FIGURES 5A, B, C, and D These figures show the effect of GSL depletion on CD4-chemokine receptor association. (A), CD4-CCR5 association. CD4 coimmunoprecipitated by anti-CCR5 antibody 5C7 from 3T3-CD4-CCR5 cells treated (+) and not treated (-) with PPMP. The gels shown are Western blots using rabbit anti-CD4 and goat anti-CCR5 antibody, respectively. The numbers below the gels represent intensity ratios determined using a Molecular Imager (Biorad, Hercules, CA). (B), CD4-CXCR4-gp120 association. CD4 coimmunoprecipitated by anti-CXCR4 antibody 4G10 from 3T3-CD4-CXCR4 treated (+) and not treated (-) with PPMP in the presence of (+) or absence of (-) $rgp120_{IIIB}$. The gels shown are Western blots using rabbit anti-CD4 and mouse anti-CXCR4 antibody respectively. The numbers below the gels represent intensity ratios determined using a Molecular Imager (Biorad, Hercules, CA). (C), Inhibition of fusion with cells expressing the R5-utilizing envelope glycoprotein (Ba-L). (D), Inhibition of fusion with cells expressing the X4-utilizing envelope glycoprotein (IIIB).

FIGURE 6This figure shows the kinetics of exposure of hydrophobic binding sites upon addition of HeLaCD4 cells to $gp120\text{-}gp41_{IIIB}$-expressing TF228 cells.

The average changes in bis-ANS fluorescence intensity of 7-13 individual cells are shown against time following addition of the TF228 cells. The arrow indicates the time at which the effector cells were touching the target cells. ( ) Untreated HeLaCD4 cells, ( ) PPMP-treated HeLaCD4 cells.

FIGURES 7A, B, and C These figures show the recovery of fusion after the addition of various glycosphingolipid fractions that were isolated from a crude human erythrocyte mixture. (A) TLC analysis of Erythrocyte glycosphingolipids: GSL were isolated as described below and 25-50 μg of total lipid was spotted on 5x20 cm silica gel TLC plate. The plate was developed in $CHCl_3$:MeOH:H_2O (65:25:4, v/v). At the end of the run, the plate was air-dried, sprayed with Bial's reagent, heated to develop the spots and scanned using a Hewlett Packard 4P scanner and photographed. GSL isolated from human (lane 1) and bovine (lane 2) erythrocytes; GSL standards are in lane 3. (B) TLC of Various GSL Fractions Purified by Silica Gel Column: The crude GSL mixture from human erythrocytes (From Fig 1A, Lane 1) was fractionated by silica gel column chromatography. GSLs in the fractions were analyzed by chromatography on silica gel TLC as in FIGURE 7A. Lanes 1-7 = fraction numbers. (C) Recovery of Fusion of $CD4^+$ Non-human Cells by GSL fractions: GP4F cells were plated on microwells and infected with vCB3 to express human CD4 on the cell surface. Liposomes containing different GSL fractions were incorporated into the membranes

of the target cells and the cells were labeled with CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine). DiO (3,3'-dioctadecyloxacarbocyanine perchlorate) -labeled TF228 cells were cocultured with GSL-supplemented target cells for 4-6 hours at 37°C. Images were collected and fusion was calculated as described below. Data are presented here of the fraction of recovery of fusion by the crude human GSL fraction.

FIGURE 8A This figure illustrates the chemical structure of globo series glycosphingolipids. (I), Glc Cer; (II), Gb2 (Gal(β1-4)Glc Cer); (III), Gb3 (Gal(α1-4)Gal(β1-4)GlcCer); and (IV), Gb4 (GalNAc((β1-3)Gal(α1 4)Gal(β1-4)Glc Cer.

FIGURE 8B This figure shows the thin layer chromatography analysis of fraction 3. GSLs were chromatographed on Silica Gel TLC plates as in FIGURE 7A and sprayed with Orcinol spray. Lane 1, Fraction 3, Lane 2, Gb3 (Top spot); Lane 3, The same neutral GSL standards as shown in FIGUREs. 7A and B.

FIGURE 8C This figure shows the results from the HPTLC Immunostaining experiments. GSLs (5μg) were chromatographed on Silica Gel HPTLC plates.
The plates were air dried and sprayed either with orcinol spray (left panel), incubated with 38.13 antibody (middle panel), or incubated with asialo-GM2 antibody (right panel). Ln 1, Fraction 3; Ln 2, Asialo-GM2; Ln 3, Gb3.

FIGURE 8D This figure shows the mass spectrum of 24:0 Gb3 detected from Fraction 3.

FIGURE 8E This figure shows the mass ion chromatogram of various molecular species of Gb3 present in Fraction 3.

FIGURE 8F This figure shows the ^{1}H NMR proton spectrum of Fraction 3 in 98:2 DMSO-d6:D2O at 298 K. Relevant peaks are labeled with arrows. Numbers in parentheses refer to the position of the particular sugar in the trisaccharide with glucose attached to the ceramide as position 1. The peak marked with a star (*) is thought to arise from minor variations in the length of the ceramide fatty acid chains.

FIGURES 9A and B This figure shows an enzymatic digestion of Fraction 3.
(A), Fraction 3 was digested with α-GalA in parallel with standard GSLs and the results analyzed by HPTLC. S: mixed GSL standards; 1: Gb2 (no enzyme); 2: Gb2 (+ α-GalA); 3: Gb3 (no enzyme); 4: Gb3 (+ α-GalA); 5: Fraction 3 (no enzyme); 6: Fraction 3 (+ α-GalA). (B), Fusion activity following addition of α-GalA-digested Fraction 3. Digested lipid was extracted, incorporated into liposomes, transferred to HeLa-CD4/GSL and fusion activity was monitored as in FIGURE 7C. Controls represent untreated Hela-CD4 cells. Gb2 was added as an additional control.

FIGURE 10 This figure shows the recovery of fusion activity by the addition of various GSLs. The lipids were incorporated into liposomes, transferred to HeLa-CD4/GSL$^-$ cells and fusion activity was monitored as in FIGURE 7C. Controls represent untreated Hela-CD4 cells. Data are from one of three similar experiments.

FIGURE 11A and B These figures show the recovery of HIV-1 fusion activity by reconstitution of PPMP-treated cells with GSLs. Lipids were incorporated into liposomes and transferred to Control and PPMP-treated GHOST-X4 and -R5 cells as described in EXAMPLE 1. Fusion activity was monitored as described using vaccinia vectors that express X4 (IIIB, vpE16), R5 (Ba-L, vcB43) and X4R5 (89.6, vDC-1) in HeLa cells. (A), GHOSTX4 cells as targets and (B), GHOSTR5 cells as targets. PPMP$^-$ GHOSTX4 and GHOSTR5 are the untreated cells; PPMP$^+$ GHOSTX4 and GHOSTR5 are the PPMP-treated cells without addition of GSL; PPMP$^+$ GHOSTX4 and GHOSTR5 + GSL are the PPMP treated cells with addition of the indicated GSL.

DETAILED DESCRIPTION OF THE INVENTION

In the disclosure below, the inventors describe their discovery that specific GSLs (e.g., Gb3 and GM3) are involved in HIV-1 entry and that HIV-1 infection can be inhibited by providing agents that interfere with the interaction of these GSLs with gp120-gp41. In a first set of experiments, the inventors observed that treatment of GHOST cells with a GSL synthesis inhibitor (e.g., 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP)) resulted in a marked reduction of GSLs on these cells and concomitantly inhibited HIV-1 entry without a change in the cell's phospholipid or cholesterol composition or expression of CD4, CXCR4, or CCR5 receptors. Further, the inventors found that PPMP treated GHOST cells retained their ability to respond the the CXCR4 ligand SDF1-α, including the ability of this ligand to induce calcium mobilization.

To characterize the specific GSLs that were involved in HIV-1 entry, individual component(s) of a human erythrocyte GSL mixture were isolated by fractionation on a silica gel column and incorporated into the membranes of CD4$^+$ cells. GSL fraction-supplemented target cells were then examined for their ability to fuse with TF228 cells expressing HIV-1_{LAI} envelope glycoprotein. One GSL fraction, Fraction 3, exhibited the highest recovery of fusion following incorporation into CD4$^+$ non-human and GSL-depleted HeLa-CD4$^+$ cells (HeLa-CD4$^+$/GSL$^-$). Fraction 3 was characterized by mass spectrometry, NMR spectroscopy, enzymatic analysis and immunostaining with an anti-Gb3 antibody and was found to be Gal (α1-4) Gal (β1-4) Glc-Ceramide (Gb3). Addition of Fraction 3 or Gb3 to HeLa-CD4$^+$/GSL$^-$ cells recovered fusion, but addition of GalCer, GlcCer, GM3 ((NeuNAc (α2-3) Gal (β1-4) Glc (β1-1) Cer)), Gb2 ((Gal (β1-4) GlcCer)), Gb4 (Globoside), GD3 ((NeuNAc (α2-8) NeuNAc (α2-3) Gal (β1-4) Glc (β1-1) Cer)) or α-galactosidase A-digested Fraction 3 had no effect. Further, the inventors examined whether GSLs have strain-specific effects by testing various isolates for their ability to fuse with GSL-depleted GHOST X4 or R5 cells. In these experiments the inventors discovered that GSLs, such as Gb3 and to a lesser extent GM3, are involved in the entry of a broad range of HIV-1 isolates.

Several embodiments described herein involve biotechnological tools, diagnostics, and components to prophylactic and therapeutic agents, which modulate the interaction of gp120-gp41 with GSLs. In some embodiments, GSLs and parts thereof, with or without other molecules (e.g., CD4, CCR5, or CXCR4), are joined to supports, which can be organic or inorganic structures that display a plurality of these molecules so as to create a multimerized agent. Some aspects of these embodiments include linkers that separate the GSL or receptor molecules from the support so as to alleviate steric hindrance and/or facilitate attachment of the molecules. Additional embodiments include multimeric supports comprising multiple GSLs and/or receptor molecules that are fused in tandem and joined to a support. One tool provided herein concerns the use of GSLs to enhance fusion to a support having gp120-gp41, including but not limited to a cell or liposome. Other embodiments concern the use of GSLs, such as Gb3, to prepare and isolate HIV particles, to remove HIV particles from solutions, including but not limited to body fluids such as blood, and to manufacture dialysis filiters that can rapidly remove HIV particles from a subject. Aspects of the invention also include diagnostic tools that detect the presence of HIV infection based on the interaction with a GSL and approaches to identify prophylactic and therapeutic agents that modulate the interaction of GSLs and gp120-gp41. Several methods to inhibit HIV infection and formulate therapeutic and prophylactic agents are also described in this disclosure. In the section below, the inventors describe experiments that provide evidence that GSLs are involved in critical interactions with gp120-gp41 and, thereby, modulate HIV-1 fusion and entry.

Inhibition of GSL biosynthesis blocks target cell susceptibility to HIV-1 infectivity of a broad variety of HIV-1 isolates

The synthesis of most GSLs begins with glucosylation of ceramide to form glucosylceramide (GlcCer), the precursor for hundreds of different glycosphingolipids. (Ichikawa and Hirabayashi, *Trends. Cell Biol.*, 8:198-202 (1998)). This cerebroside is synthesized from uridine diphosphate-glucose and ceramide by the glucosyltransferase, GlcCer synthase. One way to better understand the functions of GSLs is to selectively inhibit cellular glucosylceramide formation. Radin and coworkers have synthesized a variety of specific inhibitors of GlcCer synthase. (Abe et al., *J. Biochem* (Tokyo), 111:191-196 (1992)). The inventors have used one of these, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP), to examine the role of glycosphingolipids in HIV-1 entry. In order to examine susceptibility to HIV-1 infection, the inventors used GHOST(3) cell lines that constitute an HIV-1 or HIV-2 indicator cell panel whose individual lines are engineered to express a Tat-dependent green fluorescent protein (GFP) reporter cassette in conjunction with CD4 and a specific chemokine receptor. (Cecilia et al., *J. Virol.*, 72:6988-6996 (1998)). To examine the role of GSL in HIV-1 infection, the inventors used env-complemented HIV-1 pseudotypes and $CD4^+$ target cells that stably express CXCR4 (GHOSTX4), CCR5 (GHOSTR5), or CCR3, CXCR4 and CCR5 (GHOST345). HIV-1 infection resulted in GFP expression that was monitored by counting stained cells in the fluorescence microscope.

FIGURE 1 shows the results for the pseudotypes containing env expression vectors for HIV-1 HXB2, 89.6, ADA, BaL, JRCSF. In all cases, treatment of GHOST cells with PPMP inhibited HIV-1 infection. The PPMP treatment

resulted in a marked reduction of GSLs in these cells (FIGURE 2). Treatment of cells with PPMP did not affect entry of viruses pseudotyped with the envelope glycoprotein of Vesicular Stomatitis Virus (VSV) or amphotropic Murine Leukemia Virus (A-MLV), however. Furthermore, the phospholipid and cholesterol composition were unchanged following PPMP treatment. Moreover, a significant change in CD4, CXCR4 and CCR5 expression following treatment of these cells with PPMP was not seen (FIGURE 3).

Chemokines and their seven-transmembrane domain G protein-coupled receptors constitute a large and highly differentiated signaling system involved in many biological processes, including development, hematopoiesis, angiogenesis, and regulation of specific leukocyte trafficking. (Baggiolini, *Nature,* 392:565-568 (1998)). The activity of the chemokine receptors has been examined by monitoring chemotaxis in response to specific ligands. FIGURE 4 shows that treatment of GHOST X4 cells with PPMP, which inhibited HIV-1 entry, did not affect the cell's ability to respond to SDF1-, a ligand for CXCR4. Additionally, the ability of SDF1- to trigger Ca^{2+} mobilization in the cells was also unaffected by pre-treatment with PPMP.

It has been demonstrated that CXCR4 can directly associate with the complex between CD4 and the HIV-1 envelope glycoprotein providing evidence that the complex between these three molecules plays a critical role in the initial stages of the entry process. (Lapham et al., *Science,* 274:602-5 (1996)). More recently, it has been shown that cell surface CD4 associates with CCR5 in the absence of gp120, or other chemokine-receptor- or CD4-specific ligands, and that there exists a functional correlation between this association and HIV-1 envelope glycoprotein-mediated fusion. (Xiao et al., *Proc. Natl. Acad. Sci. U.S.A.*, 96:7496-7501 (1999)). Since these molecules can be associated in membrane domains, the inventors set out to determine whether GSL's are involved in this association.

In these experiments, the inventors used NIH3T3CD4X4 and NIH3T3CD4R5 cells because co-immunoprecipitation of CD4 with CXCR4 or CCR5 in these cells yielded a better signal in Western blots. FIGURE 5 shows that treatment of NIH3T3CD4X4 and NIH3T3CD4R5 with PPMP for 7 days reduced fusion yields with cells expressing HIV-1 gp120-gp41 of the appropriate specificity. The co-immunoprecipitation data show that treatment of these cells with PPMP had no effect on association of CD4 with CXCR4 or CCR5. Moreover, treatment with PPMP did not affect the amount of gp120-induced co-immunoprecipation of CD4 and CXCR4. These results provide evidence that the formation of the trimolecular gp120-CD4-CXCR4 complex, which presumably occurs at an early stage in the fusion cascade,[20] is not dependent on the presence of GSL in the target membrane.

In a previous experiments, the inventors continuously monitored conformational changes of cell surface-expressed HIV-1 gp120-gp41 in situ using 4,4-dianilino-1,1-binaphthyl-5,5-disulfonic acid (bis-ANS), a fluorescent probe that binds to hydrophobic groups. (Jones et al., *J. Biol. Chem.,* 273:404-409 (1998)). These conformational changes, which lead to membrane fusion, were found to be highly cooperative, requiring both CD4 and an appropriate chemokine receptor. Reported here for the first time, the inventors have used the bis-ANS technique to monitor the interactions between gp120-gp41-expressing cells and $CD4^+$ / $CXCR4^+$ target cells, which are GSL-depleted and fusion incompetent. FIGURE 6 shows the results of these experiments, which demonstrate that the GSL-depleted cells failed to produce a response in the bis-ANS assay. These observations taken together with the co-

immunoprecipitation experiments (FIGURE 5) demonstrate that while the GSLs have no effect on the intrinsic associations between individual molecules of CD4 and CXCR4 or CCR5, they are necessary to trigger the supramolecular associations and massive conformational changes in the envelope glycoprotein required for membrane fusion.

Identification of GB3 as a cofactor that promotes CD4-dependent HIV-1 fusion

To isolate the cofactor that mediates CD4-dependent HIV-1 fusion and entry, the inventors separated and fractionated individual components of a human erythrocyte GSL mixture using silica gel column chromatography. (See FIGURE 7A, lane 1). Total GSLs were extracted from human erythrocytes. (Puri et al. *BBRC* 242:219-225 (1998). The GSL mixture was further separated on a silica gel column using a $CHCl_3$:MeOH solvent mixture of increasing polarity and seven fractions were taken. The solvents were removed under vacuum and the components of individual GSL fractions were detected by chromatography on silica gel thin layer chromatography (TLC). Bial's reagent, which detects sugars, glycosphingolipids, sulfolipids and gangliosides, was used to detect the GSL on TLC plates. The relative mobility of these GSL fractions on silica gel TLC plates is shown in FIGURE 7B. Each fraction of the seven total GSL fractions collected was then individually prepared and incorporated into the membranes of fusion-incompetent cells by liposome-mediated transfer.

Liposomes containing egg phosphatidylcholine: egg phosphatidylethanolamine: GSLs (3:1.5:1, w/w/w, 0.9 mg/ml total lipid) were prepared in phosphate buffered saline without Ca^{2+} and Mg^{2+} by extrusion through a 0.2 μm filter using an extruder (Lipex Biomembranes, Inc., Vancouver, BC). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). The fusion incompetent cell line GP4F/CD4$^+$ was obtained by infecting GP4F cells with the vaccinia recombinant vCB3 to express human CD4. Liposomes (1 ml) were allowed to bind to GP4F/CD4$^+$ cells and liposome-cell fusion was induced by brief exposure (60 sec.) of the cells to pH 5.1 followed by incubation in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

To monitor fusion, the GP4F/CD4$^+$ cells were labeled with the cytoplasmic dye CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) 10μM, ex/em 541/565 nm) for 2-4 hr. at 37^{0}C and cells expressing gp120-gp41$_{LAI}$ (TF228 cells) were labeled with either the fluorescent membrane probe DiO ((3,3'-dioctadecyloxacarbocyanine perchlorate), ex/em 484/501)) or aqueous probe calcein-AM (ex/em 496/517 nm). GP4F/CD4+ cells were then cocultured with TF228 cells for 2-6 hours at 37^{0}C to allow for fusion. Tissue culture media was purchased from Gibco BRL (Life Tech, Gaithersburg, MD) and other reagents were from SIGMA Chemical Co. (St Louis, MO). Fluorescent probes were obtained from Molecular probes (Eugene, OR).

After fusion, the cells were examined using an Olympus IX70 inverted microscope using U-MNG filter cube for the rhodamine probes and the U-MNIBA filter cube for the other probes. (Puri et al. *BBRC* 242:219-225 (1998). Image analysis for dye mixing was performed with Metamorph image analysis software (Universal Imaging Corp., West Chester, PA). The total number of cells positive for each dye was scored. Bright field images were used to

distinguish false positives where labeled cells were lying over one another without actual fusion. The extent of fusion was calculated as:

% fusion = 100 x [number of cells positive for both dyes] /
[number of cells positive for CMTMR].

FIGURE 7C shows fusion activity with GSL-supplemented CD4$^+$ GP4F cells. Fraction 3-enriched target cells showed the highest recovery of fusion while Fraction 6 also showed higher recovery compared to other fractions. Addition of Fractions 1,2,4,5 and 7 did not result in significant fusion. As can be seen in FIGURE 7B, Fraction 3 is one of the minor components in the crude GSL mixture.

The fractions isolated from the human erythrocyte GSL mixture were further characterized by mass spectrometry after separation by TLC. (Kundu, S.K. Methods Enzymol. 72, 185-204 (1981)). Electrospray LC/MS analysis was performed using a Hewlett-Packard 5989B mass spectrometer with Analytica electrospray source using the same parameters as described previously. (Kim et al. AnalChem. 66, 3977-3982 (1994)). Samples and known standards were injected onto a C-18 HPLC column (Phenomenex, 5 , 2.0 x 150 mm) and separated using the mobile phase consisting of water:MeOH with 0.5% NaOH:hexane changing linearly from 12:88:0 to 0:98:2 in 7 min. after holding at the initial composition for 3 min., at the flow rate of 0.3 ml/min.. The final mobile phase composition was maintained for 15 min.. Standards were obtained from Matreya, Inc. (Pleasant Gap, PA).

Resorcinol spray analysis of the TLC indicated that of the seven fractions collected, only Fractions 5 and 6 contained acidic GSLs. Furthermore, as shown in FIGURE 7A, lane 2, Fraction 3 was not present in a bovine GSL mixture that is consistent with previous observations that addition of GSLs from bovine erythrocyte membranes failed to rescue gp120-gp41-mediated fusion. (Puri et al. *BBRC* 242:219-225 (1998). Silica gel TLC analysis of Fraction 3 (FIGURE 8B, lane 1) showed a single component (>99% pure) with relative mobility very similar to that of Gb3 (FIGURE 8B, lane 2). Neither Fraction 3 nor Gb3 reacted with resorcinol spray reagent indicating that Fraction 3 is a neutral GSL. The chemical structure of GSLs of the "Globo" series is shown in FIGURE 8A. Electrospray LC/MS analysis of Fraction 3 in comparison to known standards indicated that this fraction contained mainly ceramide trihexosides. They were detected as natriated molecules (M+Na+) as is shown for 24:0-trihexoside as an example in FIGURE 8D. FIGURE 8E shows mass ion chromatograms of Fraction 3 GSL containing 24:0, 22:0 or 24:1 fatty acyl moieties and their hydroxy derivatives. Presence of these fatty acids was also confirmed by the gas chromatographic analysis after transmethylation.

NMR analysis corroborated the data obtained from enzymatic hydrolysis (see below) and mass spectrometry. NMR spectroscopy was performed on a Bruker AMX spectrometer at 500 MHz. Data for ^{1}H 1-dimensional spectra were collected at different temperatures from 288 - 323 K controlled by a Eurotherm® variable temperature unit with an accuracy of ±0.1 K. The 90 pulse width was 10.85 msec and the sweep width was 5000 Hz. The spectra were processed with Gaussian multiplication (lb = -1, gb = 0.1). Two-dimensional total correlation spectroscopy (TOCSY)

was performed with the standard pulse sequence and the data were collected with a sweep width of 4466 Hz in both dimensions and a spin lock mixing time of 65 msec as described. (Bax et al. J. *Magn. Reson.* 55:301-315. (1983)).

FIGURE 8F shows the ^1H spectrum of Fraction 3 in 98:2 DMSO-d_6/D_2O; the relevant peaks are labeled with arrows. Only one amide proton was observed (ceramide) and no acetate peaks were present confirming the absence of amino sugars in the head group. In addition, there were no indications of the presence of neuraminic acid units in the molecule in the proton spectrum. Three anomeric protons were observed and their spin systems were traced by total correlation spectroscopy (TOCSY). Coupling constants measured for the anomeric proton doublets indicated two β-linked (7.6 and 7.7 Hz) and one α-linked (3.6 Hz) sugar groups. Data for ^{13}C also showed the presence of three anomeric carbons and the one bond $^1J_{CH}$ coupling constants (measured in an HMQC[1] spectrum) consistent with the stereochemical assignments of the anomeric centers.

Fraction 3 was then acetylated with acetic anhydride/pyridine in a 1:2 ratio for 24 hr at room temperature. The reagents were evaporated under reduced pressure and the residue was taken up in water and extracted with ethyl acetate (3x). The combined organic extracts were washed with water and brine, dried by filtration through a pad of sodium sulfate. The crude peracetate was then purified on a silica gel column using a gradient of CH_3OH (0 -3%) CH_2Cl_2.

Exhaustive acetylation of Fraction 3 and purification yielded a compound with 11 acetate methyl groups present in the ^1H NMR spectrum. This number of free hydroxyl groups in the molecule was consistent with a trihexose-containing ceramide glycoside. In addition, comparison of the NMR data obtained for Fraction 3 with that of an authentic sample of ceramide trihexoside (Gb3) (top spot), and with the reported chemical shifts and coupling constants of synthetic Gb3 showed the two to be virtually identical. A minor set of peaks were observed in the fatty acid region of the spectrum and these were attributed to a small percentage of material where the ceramide portion varied in the length of the hydrocarbon chain attached at the ceramide amino group (see FIGURE 8E).

To further define the carbohydrate moieties of Fraction 3, the inventors used high-performance thin-layer chromatography (HPTLC) followed by immunostaining with an anti-Gb3 antibody (38.13), an antibody known to recognize the terminal Gal (α1-4) Gal β motif. (Wiels et al., *Proc.Natl.Acad.Sci.U.S.A.* 78:6485-6488 (1981) and Nudelman et al., *Science* 220: 509-511 (1983)). Briefly, GSLs were separated on glass-backed HPTLC plates (Analtech Inc., Newwark, DE) using $CHCl_3$:MeOH:H_2O (75:21.5:3.5 v/v) as the mobile phase. The samples (5 μg per lane) were run in duplicate sets. Plates were air-dried and split into equal halves. One portion was sprayed with Bial's reagent and the other part of HPTLC plate was mechanically stabilized by a 2 min. bath in 0.1% poly-isolbutyl methacrylate in n-hexane. The dried plates were sprayed gently with phosphate buffered saline and immediately immersed into Tris-BSA buffer to block non-specific binding for 30 min. at room temperature. The plates were then treated with appropriate dilution of anti-GSL antibodies for 1 hour at room temperature. Antibody binding was detected using alkaline phosphatase detection kit (Boehringer Manneheim Corp., Indianapolis, IN).

FIGURE 8C shows immunostaining of Gb3 and Asialo-GM2 on HPTLC plates with 38.13 and an anti asialo-GM2 antibody. The 38.13 antibody reacted with Fraction 3 and Gb3 to similar extents, but not with asialo-GM2 that

contains N-acetyl galactosamine (β1-4) as the terminal sugar group (FIGURE 8C, middle panel). Conversely, the asialo-GM2 antibody only reacted with asialo-GM2 (FIGURE 8C, right panel). These results demonstrate that the polar head group of Fraction 3 bears Gal (α1-4) Gal β sugar linkages. The rabbit anti-Asialo GM2 antibody was obtained from Matreya, Inc. (Pleasant Gap, PA). Mouse anti-GalCer mAb was obtained from Boehringer Mannaheim Corp., Indianapolis, IN, and the rat anti-Gb3 (38.13) mAb was generated as described elsewhere. (Wiels et al., *Proc.Natl.Acad.Sci.U.S.A.* 78:6485-6488 (1981)

To further confirm the exact nature of carbohydrate motifs of Fraction 3, the inventors performed enzymatic analysis using α-galactosidaseA (α-GalA) that is specific for the hydrolysis of the galactose (α1-4)-galactosyl linkage found in Gb3. Dried aliquots of Fraction 3 and authentic glycosphingolipids (20 μg) were digested by incubating for 2 hours with an excess (7000 units) α-GalA in 80 mM sodium acetate, pH 4.1 containing 0.25% Nonidet-P40 and 0.25% sodium taurocholate prepared as described previously. (Kusiak et al., *J.Biol.Chem.* 253: 184-190 (1978)). Following Folch partition, the lower phase was dried and redissolved in $CHCl_3$:MeOH (2:1) and an aliquot subjected to HPTLC. (Folch et al., *J.Biol.Chem.* 225:497-509 (1957)). Spots were visualized by spraying with 0.1% 5-hydroxy-1-tetralone in 80 % sulfuric acid and scanned on a Storm 860 fluorescent scanner (Molecular Dynamics, Sunnyvale, CA) with a blue fluorescent filter set (excitation filter:450 nm; emission filter: 520 nm) and a photomultiplier tube voltage of 900 volts. (Watanabe & Mizutsa *J.Lip.Res.*, 36:1848-1855 (1998)).

FIGURE 9 (A) shows that treatment of Fraction 3 with α-GalA resulted in the formation of a product comigrating with ceramide dihexoside that probably represents lactosylceramide (Gb2). Prior to digestion, Fraction 3 migrated coincident with Gb3, which was used as positive control in this assay system. Under the conditions used here, the α-GalA preparation was tested for α and β glycosidase activity against synthetic substrates and shown to be reactive for only α-galactosyl-containing substrates. α-GalA did not cleave sugar groups from Gb2 confirming the specificity of the enzyme. Fraction 3 was efficiently hydrolyzed by ceramide glycanase resulting in the formation of ceramide and a trihexoside confirming that the sugar attached to the ceramide in Fraction 3 was glucose attached by β-linkage. Based on the structural analysis presented above it is evident that the Fraction 3 isolated from a human erythrocyte GSL mixture is a ceramide trihexoside that possesses a polar head group identical to the well-characterized Gb3 (Gal (α1-4) Gal (β1-4) Glc-Cer).

To determine whether Fraction 3/Gb3 was a cofactor for CD4-dependent HIV-1 fusion, HeLa-$CD4^+$/GSL$^-$ cells were supplemented with a variety of well-defined GSLs and fusion with HIV-1_{LAI} env expressing TF228 cells was monitored. (Puri et al. *BBRC* 242:219-225 (1998) and Abe et al. *J.Biochem.*(Tokyo) 111: 191-196 (1992)). HeLa-$CD4^+$/GSL$^-$ cells were obtained by culturing HeLa-$CD4^+$ cells in the presence of 10μM PPMP (a competitive inhibitor of ceramide:UDP glucosyl transferase), for 7 days. HeLa-$CD4^+$/GSL$^-$ cells were labeled with CMTMR for 2-4 hours at 37°C and TF228 cells were labeled with either DiO or calcein-AM, as above. PPMP, (1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol) was procured from Matreya, Inc. (Pleasant Gap, PA) and all incubation media contained the inhibitor. PPMP treatment did not alter the levels of cell surface expressed CD4 and CXCR4 as monitored by flow cytometry and fluorescence microscopy of specific mAb-stained cells.

HeLa CD4$^+$/GSL$^-$ cells were also made to express hemagglutinin by infecting HeLa CD4$^+$ cells on microwells (5×10^4 per dish) with PR/8 influenza virus (A/PR8/34/H1N1) overnight at 37°C. The HeLa CD4$^+$/GSL$^-$ cells were then treated with 5 μg/ml trypsin for 5 min. at room temperature to convert the hemagglutinin precursor to its fusogenic form. Liposomes (1 ml) were then allowed to bind to hemagglutinin-expressing HeLa CD4$^+$/GSL$^-$ cells and liposome-cell fusion was induced by brief exposure (60 sec.) of the cells to pH 5.1 followed by incubation in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Fusion of the hemagglutinin-expressing HeLa CD4$^+$/GSL$^-$ cells and TF228 cells was allowed to take place by coculturing the cells for 2-6 hours at 37°C and fusion was monitored and scored as above.

FIGURE 9 (B) shows recovery of fusion activity after addition of either Fraction 3 or Gb3 to HeLa-CD4$^+$/GSL$^-$ cells. Neither addition of α-GalA-treated-Fraction 3 nor Gb2, a precursor of Gb3, to the target cells under identical conditions rescued fusion activity, indicating that the Gal (α1-4) motif is critical. Gb4, the major neutral GSL in the human erythrocyte membrane arises from Gb3 by addition of N-acetylgalactosamine to the terminal galactose residue of Gb3 in a β1-3 linkage (see FIGURE 8A). (Yamakawa et al. Jpn. *J. Exp. Med.* 35:201-207 (1965)). Incorporation of Gb4 into the plasma membranes of HeLa-CD4$^+$/GSL$^-$ cells did not result in recovery of fusion with TF228 cells (FIGURE 10). This result established that the terminal α-galactose residue of Fraction 3/Gb3 must be at the terminal position for HIV-1 env-mediated fusion. While others have shown that GD3, Gb2 and GM3 specifically interact with HIV-1, FIGURE 10 clearly reveals that Gb3 is the cofactor necessary for CD4-dependent fusion and entry of HIV-1. (Yahi et al. *J.Virol.* 66:4848-54 (1992) and Delezay, et al. Biochemistry 35, 15663-15671 (1996)).

In order to examine possible strain-specificity of Gb3, the inventors tested various isolates for their ability to fuse with GSL-depleted GHOST X4 or R5 cells. FIGURES 11A and 11B show the specificity of these cells for the envelope glycoproteins of their respective HIV-1 isolates, and their inhibition by pre-treatment of the target cells with PPMP. Although Gb3 appeared to be the most potent GSL in restoring the fusion activity of CXCR4 and/or CCR5-utilizing envelope glycoproteins, some enhanced activity over background was also seen upon reconstitution with GM3.

In the experiments described above, the inventors demonstrate that glycosphingolipids are involved in the entry of a broad range of HIV-1 isolates into cell lines expressing CD4, CXCR4 and/or CCR5. Evidence of this discovery is provided by the observation that the inhibition of the synthesis of Glucosylceramide (GlcCer), that serves as the precursor for hundreds of different glycosphingolipids (Ichikawa and Hirabayashi, *Trends. Cell Biol.*, 8:198-202 (1998)), disrupts HIV-1 entry. GlcCer-based sphingolipids have been identified as important mediators of a variety of cellular functions, including proliferation, differentiation, development, and cell-cell recognition. (Hakomori, *Glycobiology*, 8:xi-xix (1998)). The inventors have demonstrated that cell lines cultured in the presence of PPMP, a competitive inhibitor of Glucosylceramide Synthase (Abe et al., *J. Biochem* (Tokyo), 111:191-196 (1992)), exhibit a reduction in overall glycosphingolipid content (FIGURE 2). The inhibition of GSL biosynthesis affected neither cell surface expression of CD4, CXCR4 and CCR5 (FIGURE. 3), nor the formation of a trimolecular gp120-CD4-chemokine receptor complex (FIGURE. 5). Moreover signaling of these cells via chemokine receptors was not altered by inhibition

of GSL biosynthesis (FIGURE. 4). However, lack of GSLs on the target membrane did block the massive conformational changes in gp120-gp41, that result from specific interactions between gp120-gp41, CD4 and chemokine receptor (FIGURE 6). Inhibition of GSL biosynthesis in the target membrane reduced fusion activity with env-expressing cells (FIGURE. 11), as well as the infection by HIV-1 from a variety of isolates (FIGURE. 1). PPMP-treatment of envelope glycoprotein-expressing cells did not affect their subsequent fusion with appropriate (untreated) target cells, indicating that the GSL effect is unidirectional. Inhibition of GSL biosynthesis did not affect entry of virus pseudotyped with the envelope glycoproteins from VSV or amphotropic MuLV (FIGURE. 1), which indicates that the GSL effect is unique for HIV-1.

The inventors have discovered that the GSLs range in their ability to restore the fusion activity of all HIV-1 isolates tested with Gb3 being preferred (e.g., Gb3 > > GM3 > GD3). In T lymphocytes, however, the monosialoganglioside GM3 represents the main ganglioside constituent of the plasma membrane (72% of total gangliosides); Gb3 is not detectable. Nevertheless, HIV-1 envelope glycoprotein- mediated cell fusion is inhibited following PPMP treatment of SupT1 cells (a T cell line) and other cell lines normally devoid of Gb3. Although addition of Gb3 to GSL-depleted cells recovers fusion, even in backgrounds where Gb3 is normally absent, other glycosphingolipids, such as GM3, can fulfill the necessary role in mediating HIV-1 fusion.

In recent studies, investigators postulate that sphingolipid and cholesterol-rich domains exist as phase-separated "rafts" in the membrane, which serve as sites enriched in signal transduction assemblies. (Simons and Ikonen, *Nature,* 387:569-572 (1997); Brown and London, *Annu. Rev. Cell Dev. Biol.,* 14:111-136 (1998)). According to a model proposed by Fantini et al., for example (Hammache et al., *J. Virol.,* 73:5244-5248 (1999)), the glycosphingolipids recognized by both CD4 and gp120 induce the formation of a trimolecular complex of CD4, glycosphingolipid and gp120 in such rafts. The observation that CD4 is found in GM3-enriched domains on the lymphocyte plasma membrane (Sorice, et al., *J. Lipid Res.,* 38:969-980 (1997); Millan et al., *Tissue Antigens,* 53:33-40 (1999)) is consistent with this hypothesis. Although the interaction between CD4, gp120, and the GSL is consistent with the data presented above, a slightly different model is more appropriate:

Not wanting to limit the scope of any embodiment of the invention to any particular mechanism or mode of action, the following theory is offered only as one possible mechanism by which HIV-1 entry occurs. Accordingly, the inventors believe that CD4 and chemokine receptors are associated before gp120 interaction and that this association is not dependent on the presence of GSL (See FIGURE. 5). These complexes are enriched in rafts, leading to a higher local concentration within a cell-surface microdomain. In the absence of GSL, the trimolecular gp120-CD4-chemokine receptor complexes are still formed (FIGURE. 5), but the massive conformational changes required for fusion do not occur (FIGURE. 6). Further, the inventors propose that secondary interactions between the V3 loop of gp120 and the polar heads of GSL molecules lead to the conformational changes in gp120-gp41 that allow for dissociation of gp120 from gp41 and that this step enables gp41 to form the viral hairpin (Simons and Ikonen, *Nature,* 387:569-572 (1997)) that promotes assembly of the gp41 molecules into the fusion machine. In the following discussion, the inventors

disclose several embodiments that concern the modulation of GSL interactions with gp120-gp41 so as to treat and prevent HIV-1 infection.

Embodiments that modulate interactions between gp120-gp41 and glycosphingolipids

Several embodiments of the invention involve biotechnological tools, diagnostics, prophylactics, therapeutics and methods of use of the foregoing that exploit interactions between gp120-gp41 and glycosphingolipids so as to treat and prevent HIV-1 infection. These embodiments desirably provide GSLs in such a form or in such a way that a sufficient affinity for gp120-gp41 is achieved. While a natural monomeric agent (that is, an agent that presents a discrete molecule, thus, carrying only one binding epitope or domain) can be sufficient to achieve a desired response, a synthetic agent or a multimeric agent (that is, an agent that presents multiple molecules, thus, having several binding epitopes or domains) often times can elicit a greater response. It should be noted that the term "multimeric" refers to the presence of more than one molecule on an agent, for example, several individual molecules of Gb3 joined to a support, as distinguished from the term "multimerized" that refers to an agent that has more than one molecule joined as a single discrete compound molecule on a support, for example several molecules of Gb3 joined to form a single compound molecule that is joined to a support.

The synthesis of several synthetic derivatives and multimers of Gb3 is straightforward and is known. (Armstrong et al. *J. Infectious Diseases* 171:1042-1045 (1995)). In addition to natural or synthetic Gb3, the natural or synthetic carbohydrate head group of the Gb3 trihexoside without ceramide can be used. A multimeric form of natural or synthetic Gb3 can be advantageous for many applications because of the ability to obtain an agent with higher affinity for HIV-1. Thus, one aspect of the invention contemplates using synthetic monomeric Gb3 and/or multimeric natural or synthetic Gb3. Other aspects of the invention include the use of natural or synthetic ceramide dihexosides and ceramide trihexosides that are composed of sugar combinations different than that of Gb3.

A multimeric GSL (synthetic or natural) can be obtained by coupling the GSL or, for example, the trihexoside head group of Gb3, to a macromolecular support. A "support" may also be termed a carrier, a resin or any macromolecular structure used to attach or immobilize a GSL or trihexoside head group. The macromolecular support can have a hydrophobic surface that interacts with the ceramide of Gb3 by hydrophobic non-covalent interaction. The hydrophobic surface of the support can be, for example, a polymer such as plastic or any other polymer in which hydrophobic groups have been linked such as polystyrene, polyethylene or polyvinyl. Alternatively, Gb3 or the trihexoside head group can be covalently bound to carriers including proteins and oligo/polysaccarides (e.g. cellulose, starch, glycogen, chitosane or aminated sepharose). In these later embodiments, a reactive group on Gb3 or the trihexoside head group, such as a hydroxy or the amino present in the ceramide, can be used to join to a reactive group on the carrier so as to create the covalent bond. Embodiments also can comprise a support with a charged surface that interacts with the GSL of the invention. Additional embodiments concern a support that has other reactive groups that are chemically activated so as to attach a GSL of the invention. For example, cyanogen bromide activated

matrices, epoxy activated matrices, thio and thiopropyl gels, nitrophenyl chloroformate and N-hydroxy succinimide chlorformate linkages, or oxirane acrylic supports can be used. (SIGMA).

Further, the support can comprise inorganic carriers such as silicon oxide material (e.g. silica gel, zeolite, diatomaceous earth or aminated glass) to which GSL, Gb3, or the trihexoside head group is covalently linked through a hydroxy, carboxy or amino group of the ceramide moiety of Gb3 and a reactive group on the carrier. Thus, in appropriate contexts, a "support" can refer to the walls or wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, sheep (or other animal) red blood cells, Duracyte® artificial cells, and others. Inorganic carriers, such as silicon oxide material (e.g. silica gel, zeolite, diatomaceous earth or aminated glass) to which the GSL of the invention is covalently linked through a hydroxy, carboxy or amino group and a reactive group on the carrier are also embodiments. Carriers for use in the body, (e.g., for prophylactic or therapeutic applications) should be physiological, non-toxic and preferably, non-immunoresponsive. Such carriers include, but are not limited to, poly-L-lysine, poly-D, L-alanine and Chromosorb® (Johns-Manville Products, Denver Co.). Gb3 conjugated Chromosorb® (Synsorb-Pk) has been tested in humans for the prevention of hemolytic-uremic syndrome and was reported to retain the ability to bind verotoxin after passing through the gut without adverse reactions. (Armstrong et al., *J. Infectious Diseases*, 171:1042-1045 (1995)).

In other embodiments, linkers, such as λ linkers or biotin-avidin (or streptavidin), of an appropriate length are inserted between the GSL and the support so as to encourage greater flexibility and thereby overcome any steric hindrance that is presented by the support. The determination of an appropriate length of linker that allows for optimal interaction with gp120-gp41, is made by screening the GSL of the invention, e.g., Gb3 joined to a support, having varying length linkers in the fusion assays or infection assays or both described herein.

A composite support comprising a GSL and other receptors, such as CD4 and/or a chemokine receptor, are also embodiments of the invention. A "composite support" can be a carrier, a resin, or any macromolecular structure used to attach or immobilize a GSL and CD4 or a GSL, CD4 and a chemokine receptor. Composite supports can provide higher affinity for HIV-1 and can be particularly useful for some aspects of the invention. CD4/Gb3/CXCR4 composite supports, for example, are designed to mimic the plasma membrane surface presented to HIV-1 and can provide optimal binding. Composite supports can be constructed from cell membranes comprising CD4, chemokine receptors and Gb3 or from isolated CD4, chemokine receptors and Gb3. The overexpression of CD4 and CXCR4 in tissue culture has been demonstrated. (Schnell et al., *Cell* 90:849 (1997)). By one approach, recombinant vaccinia viral constructs can be used to infect Gb3 containing cells to generate plasma membranes for use in preparing the composite support. The CD4, Gb3 and chemokine receptors can be coupled to the support by utilizing hydrophobic interactions or covalent linkages formed through reactive groups, as above.

The composite supports are also constructed by utilizing hydrophobic interactions and covalent linkages formed through reactive groups, as detailed above. Further, linkers, such as λ linkers, of an appropriate length between the GSLs and the support are inserted in some embodiments so as to encourage greater flexibility in the

molecule and overcome steric hindrance. The determination of an appropriate length of linker that allows for optimal interaction with gp120-gp41 is made by screening, as described above.

In other embodiments, the multimeric and composite supports discussed above have attached multimerized GSLs so as to create a "multimerized-multimeric support" or a "multimerized-composite support". An embodiment of a multimerized agent is obtained by creating an expression construct having two or more nucleotide sequences encoding the molecule joined together using conventional techniques in molecular biology. The expressed fusion protein is one embodiment of a multimerized agent and is then joined to a support. A support having many such multimerized agents is termed a multimerized-multimeric support. Linkers or spacers between the domains that make-up the multimerized agent and the suport can be incorporated for some embodiments and optimally spaced linkers can be determined using the approaches described above. In a similar fashion composite-multimerized-multimeric supports with and without linkers can be constructed by joining more than one different multimerized GSL or chemokine receptor.

In one facet of the invention, GSLs are used as a tool for biotechnological purposes. The fusion of a virus, cell or liposome having gp120-gp41 with a $CD4^+$ cell can be enhanced by providing Gb3 to the cell in a manner that allows the cell to incorporate the ceramide trihexoside in its plasma membrane. By adding more Gb3 coreceptor to the $CD4^+$ cell than would normally be present, a virus, cell or liposome having gp120-gp41 can more efficiently fuse with the $CD4^+$ cell. This embodiment would be particularly useful for establishing in vitro cultures of HIV-1 and can have several applications in the fields of viral transfection, monoclonal antibody production and the intracellular delivery of macromolecules.

There can be many ways to obtain cells with high concentrations of Gb3 or any other ceramide in their plasma membranes. Liposome-mediated transfer, as described above, is one approach to deliver Gb3 to the plasma membrane of a cell. Alternatively, mutant cell lines can be generated by known methods and selected for according to the amount of Gb3 or another GSL present in their cell membranes, as determined by FACS, immunocytochemistry, or the fusion assays presented above. Repetitive cycles of FACS sorting, can also allow enrichment of populations of cells that have large quantities of Gb3 or another GSL on their plasma membranes. Methods of inducing cells to produce greater quantities of Gb3 can also include the over expression of genes involved in Gb3 synthesis or the disruption of genes that regulate the synthesis of Gb3. The genes involved in the synthesis and regulation of Gb3 are known and the making of expression constructs or knockout constructs is routine.

Cells expressing CD4 and having a high concentration of Gb3 in their plasma membrane will demonstrate enhanced fusion (i.e. %fusion) with viruses, cells or liposomes that have gp120-gp41 on their surface. Gb3 fusion enhancement can be used to efficiently form cell hybrids for the production of monoclonal antibodies and can be exploited to optimize viral transfection. Further, Gb3 fusion enhancement can be used to efficiently transfer liposomes containing macromolecules, such as drugs, antibodies, proteins, nucleic acids or virus. Additionally, embodiments of the invention are aimed at improving liposome mediated delivery of an agent to specific cell types in a multicellular organism. For instance, consider that HIV-1 infected T-cells often express gp120-gp41 on their cell surface and can passively infect non-infected T-cells by gp120-gp41 and CD4/Gb3 mediated cell-cell fusion. In this case, the delivery

of a Gb3 bearing liposome containing an agent that would selectively inhibit HIV replication in the gp120-gp41 expressing T-cells would be therapeutically beneficial.

It is further contemplated that embodiments of the invention can be used for the preparation and isolation of HIV-1 particles, gp120-gp41 or fusion proteins containing gp120-gp41 through the use of established affinity chromatographic methods. For this purpose, Gb3 or another GSL can be coupled to a solid support, carrier or resin, as described above, so as to create a multimeric agent and ligand binding can be accomplished in chromatographic columns, filtration, sedimentation, centrifugation or during electrophoretic separation. Alternatively, composite supports, as described above, can be used. Elution of bound ligand is accomplished by subjecting the bound support to a low pH, a high concentration of GSL or other known methods. Cleavable linkers introduced between the support and Gb3, such as disulfide bridges or cleavable proteins, as known by those of skill in the art, can be used in the alternative. In this way, bound HIV-1 particles, gp120-gp41 or fusion proteins containing gp120-gp41 can be retrieved from the GSL solid support in their native state.

Immobilized Gb3, a multimer of Gb3 or a multimer of Gb3 head groups on a support can also be used to purify HIV-1 contaminated solutions including human blood or plasma. Similar to the approach above, an HIV contaminated solution is applied to a Gb3 resin or a composite resin and is allowed to bind for a sufficient time. Successive passes over virgin Gb3 or composite resin (i.e. Gb3 or composite resin that has not been subjected to a contaminated solution) can further purify the solution. Additionally, the level of contamination of the solution can be monitored by various immunoassays described above or other known techniques. In addition to clearing HIV-1 from the contaminated solution, HIV-1 infected T-cells that express gp120 will be bound to the Gb3 resin, thus, eliminating passive infection mediated by T-cell fusion.

Another aspect of the invention involves the use of an immobilized Gb3 or composite support as a dialysis filter to remove HIV-1 and cells expressing gp120-gp41 from a patient's blood. By this method, a modified dialysis filter cartridge (a support comprising immobilized Gb3) is used to bind and remove HIV-1 and cells expressing gp120-gp41 from a patient suffering from HIV infection and/or AIDS. Several types of dialysis machines and dialysis filters are known in the art and can be modified to meet this aspect of the invention. (See e.g. Introduction to Dialysis 2nd ed., Cogan ed. (1991)). One particularly desirable design is a portable hemodialysis machine, the REDY Sorbant Hemodialysis System (REDY 2000), manufactured by Organon-Teknika Corp. (Durham, NC). (Dialysis Therapy 2nd ed., Nissenson and Fine eds., Shapiro, REDY Sorbant Hemodialysis System p. 146-149 (1993)). The REDY 2000 uses a sorbant cartridge filter containing two layers of activated charcoal, a layer of urease, a cation exchange layer and an anion exchange layer. (Id.).

Embodiments of the invention use a similarly designed hemodialysis system wherein a cartridge comprising at least one layer of Gb3 is included. In an alternative embodiment, a hemodialysis system wherein a composite support is included in the cartridge is contemplated. The Gb3 support or the composite support-based hemodialysis systems will significantly clear HIV-1 virus and T-cells expressing gp120-gp41 from the blood of an infected patient in

roughly four hours. These aspects of the invention also have the added advantages that they are portable, relatively inexpensive, generate minimal hazardous waste and can be used by a single patient in the privacy of their home.

Other embodiments involve diagnostic tools. For diagnostic purposes, viral particles present in secretions from infected patients or other biological samples can be exposed to a Gb3 or composite support. The support material in this case can be a carrier such as a dipstick, immunological vial or test card. After binding, the Gb3 or composite support is carefully washed and the presence of HIV-1 can be detected by standard techniques including but not limited to Western, Northern, ELISA and PCR analysis.

Still more embodiments concern the screening of potential prophylactic or therapeutic agents for their ability to prevent or treat HIV-1 infection. The following discussion details the use of gp120 and Gb3 immobilized supports to screen potential prophylactic, therapeutic and fusion enhancing agents. It will be appreciated from this discussion that immobilized supports comprising any other GSL can be made and used according to the methods that follow. In one embodiment then either gp120 or Gb3 is immobilized to a support, as described above. Alternatively a composite support or another GSL-based support is used. To test the effectivity of a prophylactic agent, the agent is added to the immobilized gp120, Gb3 or composite support and, subsequently, the complementary molecule (i.e. Gb3 complements gp120 and vice versa) is added. A sufficient time for binding is allowed and the unbound complementary molecule is washed away. The presence of bound complementary molecule is then determined by assays such as ELISA or immunolabeling. A successful prophylactic agent will prevent significant binding of the complementary molecule as demonstrated by little or no detection of bound complementary molecule by ELISA or immunolabeling.

Similarly, a potential therapeutic agent can be screened. As before, either a composite support, a Gb3 support or immobilized gp120 is used but, in this case, the complementary molecule is added simultaneously with the therapeutic agent. A sufficient binding time is allowed and the unbound complementary molecule is washed away. The bound complementary molecule that remains is detected, as above, and the ability of the therapeutic agent to competitively inhibit the binding of gp120 to Gb3, as indicated by the detection of gp120/Gb3 complex can be determined.

Alternatively the ability of a therapeutic agent to displace complexed gp120/Gb3 can be assessed. By this approach, either a composite support, a Gb3 support or immobilized gp120 is used, as before, but the complementary molecule is added prior to the addition of the therapeutic agent. A sufficient time for binding is allowed and the unbound complementary molecule is washed away. A potential therapeutic agent is then added to the complexed gp120/Gb3 and is allowed to interact for a sufficient time. The dislodged complementary molecule and unbound therapeutic agent is then washed away and the amount of bound complementary molecule that remains is determined, as above. Successful therapeutic agents of this sort will have a higher affinity to either gp120 or Gb3 so as to displace the bound Gb3/gp120 complex and the detection of little or no remaining Gb3/gp120 complex will indicate an effective therapeutic agent.

By using the methods detailed above, agents that enhance fusion can be also screened. Instead of treating the immobilized Gb3 or gp120 with a therapeutic or prophylactic agent, a potential enhancing agent is provided and

binding is determined. The enhancing agent can be added prior to, during or after the addition of the complementary molecule. The screening and determination of agents that enhance fusion can be useful for identifying and characterizing new HIV coreceptors, and other agents that improve HIV modeling, monoclonal antibody production and liposome mediated drug delivery.

In the alternative, a fusion assay similar to the one used to test the inhibition of fusion mediated by PPMP can be used to test for potential prophylactic, therapeutic or fusion enhancing agents. By one approach using this aspect of the invention, HeLa-CD4$^+$ cells labeled with CMTMR are cultured in the presence of the agent to be tested for a sufficient time then the treated HeLa-CD4$^+$ cells are contacted with fluorescently labeled TF228 cells for 2-6 hours at 37°C. Fusion is determined by microscopy, as above, and a successful prophylactic or therapeutic agent will demonstrate a reduction in the %fusion, as compared to untreated control cells, whereas, a successful enhancing agent will show an increase in the %fusion in relation to the untreated control cells. One of skill in the art will appreciate that similar assays can be performed using a virus, liposome or support comprising any other GSL or gp120 to test potential therapeutic, prophylactic or enhancing agents.

Several types of prophylactic or therapeutic agents that inhibit the synthesis, transport or availability of GSLs including Gb3 are also contemplated by the invention. In one embodiment, an inhibitor of the biosynthesis of GSLs, glucosyl ceramides and/or Gb3 is administered to a subject suffering from HIV-1 infection and/or AIDS. As a first step, such a patient is identified, and as a last step this patient is evaluated for effect.

As contemplated by the inventors, a glycosphingolipid synthesis inhibitor, more specifically an inhibitor of a glucosyl ceramide or Gb3 is administered to a subject infected with HIV-1. One such inhibitor that will efficiently inhibit the production of Gb3 and HIV-1 fusion is PPMP. Analogs to PPMP such as PPPP that demonstrate an effective inhibition of cell-cell fusion by the assays presented above are also within the scope of the invention. Further amino-sugar derivatives like N-butyldeoxygalactonojirimycin (NB-DGJ) and related analogs, particularly N-hexyl derivatives of NB-DGJ, are contemplated by the inventors. NB-DGJ is an inhibitor of the ceramide specific glucosyl transferase and has been proposed to be effective for the treatment of Gaucher's disease, a glycolipid storage disorder. (Platt et al. *J. Biol. Chem.* 269:27108-27114 (1994)).

Dosages of the GSL synthesis inhibitors can vary widely depending on the compound used and the intended use (i.e. whether for prophylaxis or treatment and the age and condition of the patient). Generally, therapeutically beneficial levels of PPMP and NB-DGJ can be from between 5 -50μM and preferably are between 5 and 20μM, advantageously 10μm. Other dosages can be calculated. The level of GSL inhibition is preferably monitored during the administration of the inhibitory agent so that the dose of inhibitor can be adjusted and a stable beneficial concentration of inhibitor in the patient is maintained. Analysis of the presence of Gb3 on patient cell plasma membranes by FACS is one approach that can be used to rapidly monitor the effectivity of Gb3 inhibition during the course of treatment.

The synthesis of GSLs, glucosyl ceramides or Gb3 can also be selectively inhibited by oligonucleotides or ribozymes. The nucleotide sequence of several enzymes involved in GSL biosynthesis is known and the manufacture

of antisense oligonucleotides or ribozymes that inhibit the transcription of GSL synthesis genes would be straight forward to those of skill in the art. By employing the assays described above, various oligonucleotide and ribozyme inhibitors can be screened for their ability to prevent cell-cell fusion. Further, a therapeutically beneficial dosage of the inhibitor can be determined, by monitoring the reduction of GSL, glucosyl ceramide or Gb3 at the surface of a patient's cells by FACS. Monitoring the levels of Gb3 on the surface of the patient's cells during the course of treatment, as above, is also advised so that GSL suppression can be sufficiently maintained. The use of antisense oligonucleotides and ribozyme agents to inhibit GSL biosynthesis has the added advantage that enzymes involved in the addition of specific sugar residues can be selectively inhibited so that HIV infection can be treated and/or prevented with little adverse consequence to the patient.

The synthesis of GSLs, glucosyl ceramides or Gb3 can also be inhibited by the intercellular introduction of monoclonal antibodies that are specific for enzymes involved in glycosphingolipid synthesis. Monoclonal antibodies directed to enzymes involved in glycosphingolipid synthesis are generated by known methods and are tested for their ability to inhibit cell-cell fusion by a slight modification of the fusion assays described above. By one approach, liposomes containing a mAb are cultured with $Gb3^+$ cells and after a sufficient time for fusion and inhibition is provided, the amount of Gb3 remaining on the cells is determined by FACS. Successful therapeutic or prophylactic mAbs will demonstrate complete inhibition of Gb3 synthesis as indicated by the failure to detect Gb3 by FACS on the cell surface over time.

Monoclonal antibodies that efficiently inhibit synthesis of GSLs, glucosyl ceramides or Gb3 in vitro are then used for prophylactic or therapeutic application in vivo. To inhibit the synthesis of GSLs in a patient suffering with HIV infection or AIDS, a liposome bearing gp120-gp41 that contains the therapeutic mAb is used so that cells having Gb3 can be selectively inhibited. As before, the dosage of the agent is determined experimentally and can be patient specific. A proper dosage is found by monitoring the inhibition of Gb3 synthesis in the patient's cells by FACS over the course of treatment. The dose of mAb is also adjusted to maintain a constant level of inhibition. In another embodiment of this aspect of the invention, several mAbs that inhibit different enzymes involved in the synthesis of GSLs, glucosyl ceramides or Gb3 are administered simultaneously so as to achieve optimal inhibition.

Alternatively, a vehicle, such as a virus, liposome, or plasmid, having a nucleic acid encoding a mAb that would sequester an enzyme involved in GSL synthesis is contemplated. By one approach, the nucleic acid present in the vehicle would include nucleotide sequence encoding a Golgi localization signal as well as sequence encoding the mAb so that the addition of sugar residues can be specifically inhibited at the Golgi. In a similar manner, mAbs that interfere with the transport of GSLs can be generated intercellularly so that the delivery of Gb3 can be trapped in the Golgi. As above, the dosage of the agent is determined experimentally and can be patient and mAb specific. A proper dosage is found by monitoring the inhibition of GSL synthesis in the patient's cells by FACS over the course of treatment. The dose of mAb is also preferably adjusted so as to maintain a constant Gb3 inhibition.

The invention further relates to the use of mAbs that bind to precursors of GSL biosynthesis so as to inhibit the production of Gb3. For example, mAbs that bind to Gb2 or another glucosyl ceramide can prevent the addition of

further sugar residues and, thus, inhibit Gb3 synthesis. By one method, these antibodies can be generated by treating the patient with a vehicle comprising a nucleotide sequence encoding the mAb and a Golgi signal sequence. As before, the dosage of the nucleic acid encoding the mAb is determined experimentally and can be patient and mAb specific. A proper dosage is found by monitoring the inhibition of Gb3 synthesis in the patient's cells by FACS over the course of treatment. The dose of mAb is also adjusted to maintain a constant level of Gb3 inhibition.

Another aspect of the invention involves the treatment of HIV infection and AIDS by administering agents that interfere with the function of gp170 -- the molecule associated with the multi-drug resistance (MDR) phenotype. The MDR phenotype is consistently associated with over expression of a 170 kD membrane glycoprotein (gp170) that is responsible for an ATP-dependent efflux of a broad spectrum of structurally and functionally distinct drugs from MDR cells. (Kane et al. J., *Bioenerg. Biomembr.* 22:593 (1990) and Riordan et al., *Pharmacol. Ther.*, 28: 51 (1985)). Known agents that modulate gp170 activity include calcium channel blockers, calmodulin inhibitors, antiarrythmics, antimalarials, lysoosmotic agents, steroids, antiestrogens, cyclic peptide antibiotics and gp170 specific antibodies. (Rittmann-Grauer et al. *Cancer Res.* 52: 1810 (1992) and Pearson et al. J. *Natl. Cancer Inst.* 83: 1386 (1991)). An additional activity of gp170 is to translate glucosyl ceramide from the cytosolic face of the Golgi where it is synthesized, in the lumen of the Golgi where additional sugar groups are added to create many GSLs including Gb3. (van Helvoort et al., *Cell* 87:507 (1996)). The inventors contemplate the administration of agents that inhibit the function of gp170, with and without the administration of the therapeutic and prophylactic agents mentioned above. The administration of therapeutically beneficial doses of gp170 inhibiting agents can be determined by the approaches that have been established above. Preferably, the level of inhibition of GSL synthesis is monitored by FACS analysis of patient's cells and the dose of gp170 inhibitor is adjusted to achieve maximal Gb3 synthesis inhibition.

Another facet of the invention includes the use of agents that inhibit the function of Gb3 or other GSLs by cleaving sugar residues on GSLs, glucosyl ceramides or Gb3. The administration of α-galactosidase can inhibit CD4-dependent-Gb3-mediated cell fusion, for example, if it is provided at an appropriate dose and in an appropriate form. The dosage of α-galactosidase can be patient specific and can be determined by the amount of agent necessary to abolish binding of the anti-Gb3 antibody (38.13), as detected by FACS. As before, periodic monitoring and adjustment of the dose of α-galactosidase is performed in accordance with the levels of MAb 38.13 detected by FACS. In the alternative, other enzymes that cleave sugars on GSLs, glucosyl ceramides or Gb3 can be used including ceramide glycanase.

Embodiments of the invention can also comprise administering an agent that binds to GSLs, glucosyl ceramides or Gb3 thereby inhibiting the binding of HIV-1 to gp120-gp41 expressing cells. There can be many agents designed by one of skill in the art to bind to Gb3, for example, so as to interfere with the binding of gp120-gp41. Several such agents have been described in the literature in relation to bacterial toxins. Some strains of Esherichia Coli produce a verotoxin that bind to Gb3 causing hemorrhagic colitis and hemolytic uremic syndrome. (Lingwood, *Tr. in Microbio.* 4:147-153 (1996)). Similarly, Shigella dysenteriae produces a Shiga toxin that recognizes Gb3 in order to mediate cell cytotoxicity. (Id.). The verotoxin family comprises many members that bind to Gb3 and cause related

diseases in various animals. (Id.). Each verotoxin has a single A subunit, which is responsible for interrupting protein synthesis in the cell, and 5 B subunits, which bind to the Gb3 receptor. (Id.). Based on x-ray crystallography, researchers postulate that each B monomer has two Gb3-binding sites. (Id.). The production of verotoxin B subunit peptides and mutants thereof that demonstrate a high affinity for the Gb3 receptor is well known. (Ghislain et al., *J. of Immunol.* 153:3655-3663 (1994)).

One aspect of this embodiment a medicament comprising a verotoxin B subunit, a mutant verotoxin B subunit or a hetero multimer comprising an inactive verotoxin A subunit and five verotoxin B subunits is provided to a patient having HIV-1 infection or AIDS. As the verotoxin medicament enters the blood stream of the patient, the B subunits or mutant B subunits will bind to accessible Gb3 and competitively inhibit the binding of gp120-gp41. Ideally, a very high affinity verotoxin B subunit is used so as to not only out-compete the binding of gp120-gp41 but also to displace already bound Gb3. Embodiments of the invention can also include a protein that is at least 60% - 99% homologous to a verotoxin. That is, embodiments of the invention can include a protein that is at least 60.0%, 61.0%, 62.0%, 63.0%, 64.0%, 65.0%, 66.0%, 67.0%, 68.0%, 69.0%, 70.0%, 71.0%, 72.0%, 73.0%, 74.0%, 75.0%, 76.0%, 77.0%, 78.0%, 79.0%, 80.0%, 81.0%, 82.0%, 83.0%, 84.0%, 85.0%, 86.0%, 87.0%, 88.0%, 89.0%, 90.0%, 91.0%, 92.0%, 93.0%, 94.0%, 95.0%, 96.0%, 97.0%, 98.0%, and 99.0% homologous to a verotoxin. Further, peptide fragments of mutant or wild-type verotoxin subunits or peptidomimetics that resemble these molecules are embodiments of the invention that can be used to interact with Gb3 or other GSLs and thereby interfere with HIV-1 entry.

To select such high affinity verotoxin-based inhibitors, in vitro binding assays or fusion assays can be performed with and without competition by gp120-gp41. By this experimental approach, verotoxin based inhibitors that have the most promise for therapeutic or prophylactic application can be selected. The dosage of verotoxin based inhibitors that will be therapeutically effective will depend on the characteristics of the inhibitor and the specifics of the patient's condition. For these reasons, the dosage is best determined and adjusted by monitoring the amount of accessible Gb3 in the patient by FACS, as described above.

Alternatively, antibodies or other agents that bind to Gb3 can be administered to a patient suffering from HIV-1 infection or AIDS. As with the binding of the verotoxin-based inhibitors, antibodies such as the mAb 38.13 or other agents such as lectins, recombinant proteins, synthetic peptides or chemicals are contemplated to competitively inhibit the binding of Gb3 by gp120-gp41. These agents are screened in vitro for their ability to inhibit binding and fusion, as above, and the dosages, adjustments and monitoring is performed as described previously.

Another embodiment involves the administration of GSLs, glucosyl ceramides or Gb3 to competitively inhibit the binding of HIV-1 or gp120-gp41 expressing cells. Synthetic or natural GSLs, glucosyl ceramides or Gb3 is administered to a patient in a physiological form so as to competitively inhibit the binding of gp120-gp41 on the plasma membrane of the patient's cells. Ideally, the GSLs are designed with higher affinity for gp120-gp41 than is exhibited by the natural monomeric molecule so that competition is skewed in favor of the inhibitor. One approach involves the use of a medicament comprising multimeric GSLs, glucosyl ceramides or Gb3. These multimeric or

multimerized molecules can be obtained synthetically or by linking reactive groups present in the ceramide tails. An agent comprising GSLs, glucosyl ceramides or Gb3 immobilized to a support is, therefore, another advantageous embodiment of this aspect of the invention. As described above, the administration of Gb3 resins to humans for the purpose of studying the prevention of Hemolytic Uremic Syndrome has been reported. (Armstrong et al., *J. Infect. Diseases* 171:1042-1045 (1995)). The administration of composite supports for the purpose of the treatment and prevention of HIV-1 infection and/or AIDS is now contemplated. More discussion of the formulation of therapeutic and prophylactic agents id provided in the section below.

The manufacture and dose of therapeutic and prophylactic agents

The active ingredients of the invention (e.g., GSLs, glucosyl ceramides, Gb3, nucleic acids and proteins) are suitable for treatment of subjects either as a preventive measure to avoid a disease or infection, or as a therapeutic to treat subjects already afflicted with disease or infection. These pharmacologically active compounds can be processed in accordance with conventional methods of galenic pharmacy to produce medicinal agents for administration to subjects, e.g., mammals including humans. The active ingredients can be incorporated into a pharmaceutical product with and without modification. Further, the manufacture of pharmaceuticals or therapeutic agents that deliver the pharmacologically active compounds of this invention by several routes are aspects of the invention. For example, and not by way of limitation, DNA, RNA, and viral vectors having sequence encoding enzymes that synthesize GSLs, glucosyl ceramides, or Gb3 are within the scope of aspects of the invention. Nucleic acids encoding enzymes can be administered alone or in combination with other active ingredients.

The compounds of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral (e.g., oral) or topical application that do not deleteriously react with the pharmacologically active ingredients of this invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols, polyetylene glycols, gelatine, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like that do not deleteriously react with the active compounds.

The effective dose and method of administration of a particular pharmaceutical formulation can vary based on the individual patient and the type and stage of the disease, as well as other factors known to those of skill in the art. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population). The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with no

toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors that can be taken into account include the severity of the disease state of the patient, age, and weight of the patient; diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Short acting pharmaceutical compositions are administered daily whereas long acting pharmaceutical compositions are administered every 2, 3 to 4 days, every week, or once every two weeks. Depending on half-life and clearance rate of the particular formulation, the pharmaceutical compositions of the invention are administered once, twice, three, four, five, six, seven, eight, nine, ten or more times per day.

Routes of administration of the pharmaceuticals of the invention include, but are not limited to, transdermal, parenteral, gastrointestinal, transbronchial, and transalveolar. Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the pharmacologically active compounds to penetrate the skin. Parenteral routes of administration include, but are not limited to, electrical or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. Transbronchial and transalveolar routes of administration include, but are not limited to, inhalation, either via the mouth or intranasally.

Compositions having the pharmacologically active compounds of this invention that are suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams, and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device ("transdermal patch"). Examples of suitable creams, ointments, etc. can be found, for instance, in the Physician's Desk Reference. Examples of suitable transdermal devices are described, for instance, in U.S. Patent No. 4,818,540 issued April 4, 1989 to Chinen, et al., herein incorporated by reference.

Compositions having the pharmacologically active compounds of this invention that are suitable for parenteral administration include, but are not limited to, pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection.

Compositions having the pharmacologically active compounds of this invention that are suitable for transbronchial and transalveolar administration include, but not limited to, various types of aerosols for inhalation. Devices suitable for transbronchial and transalveolar administration of these are also embodiments. Such devices include, but are not limited to, atomizers and vaporizers. Many forms of currently available atomizers and vaporizers can be readily adapted to deliver compositions having the pharmacologically active compounds of the invention.

Compositions having the pharmacologically active compounds of this invention that are suitable for gastrointestinal administration include, but not limited to, pharmaceutically acceptable powders, pills or liquids for

ingestion and suppositories for rectal administration. Due to the ease of use, gastrointestinal administration, particularly oral, is a preferred embodiment.

EXAMPLE 1 below describes several of the materials and methods employed in the experiments described herein.

EXAMPLE 1

Materials: Fluorescent probes were obtained from Molecular Probes (Eugene, OR), and tissue culture media were obtained from Gibco/BRL (Gaithersburg, MD). Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL), and 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP), GSLs, and the monosialoganglioside mixture were from Matreya (Pleasant Gap, PA). pNL4-3Luc r'e', pBsTAT, pCMVsREV, pNL1.5E, pHCMV-G, and pJRCSF were the gift of George Pavlakis and Margherita Rosati (NCI-FCRDC, Frederick, MD). pJRFL, pADA, pBAL, p89.6, pSV-A-MLV-env, and pHXB2 were the gift of Daniel Littman and Vineet KewalRamani (NYU, NY). The Mabs, 5C7 and 4G10 were gifts from L.Wu (Leukocyte, Inc, Cambridge, MA) and Chris Broder (USUHS, Bethesda, MD). Other reagents were from Sigma (St Louis, MO).

Cell Culture: GHOST-X4 and GHOST-R5 cells were obtained from the NIH AIDS Repository. GHOST-R3/X4/R5 (GHOST-345), NIH 3T3-CD4/X4 and NIH 3T3-CD4/R5 cells, and 293T cells were the gift of Dan Littman and Vineet KewalRamani, HeLa cells were from John Silver (NIAID, Bethesda, MD), and TF228 from Zdenka L. Jonak (Smith, Kline & Beecham, King of Prussia, PA). HeLa cells were grown in DMEM + 10% FBS (D10). NIH 3T3-CD4/X4 and NIH 3T3-CD4/R5 were grown in D10 + 3 g/ml puromycin. GHOST-X4, R5, and 345 cells were grown in D10 + 500 g/ml G418, 100 g/ml hygromycin, and 1 g/ml puromycin. All cells were grown in the presence of penicillin and streptomycin. HIV-1 envelope proteins were transiently expressed on the surface of HeLa cells using the recombinant vaccinia constructs vPE16 (IIIB, X4-utilizing) (Earl et al., *J. Virol.*, 65:31-41 (1991)), vCB43 (Broder and Berger, *Proc. Natl. Acad. Sci.U.S.A.*, 92:9004-9008 (1995)) (Ba-L, R5-utilizing), and vDC-1 (89.6, X4/R5-utilizing) (Chabot et al., *J. Virol.*, 73:6598-6609 (1999)) as described previously. (Jones et al., *J. Biol. Chem.*, 273:404-409 (1998)). Cells were grown in medium containing PPMP for at least 7 days before being used in a fusion assay. GHOST-X4, R5, and 345 cells were grown in medium containing 10 M PPMP; NIH 3T3-CD4/X4 and NIH 3T3-CD4/R5 cells were grown in medium containing 7.5 M PPMP.

Infection Assays: Single-round infection assays using viral particles containing genomes with a defective env, were conducted according to the protocol of KewalRamani and coworkers. (Cecilia et al., *J. Virol.*, 72:6988-6996 (1998)). Briefly, viral stocks were prepared by cotransfecting a plasmid containing the NL3-4 genome (pNL4-3Luc r'e') (Connor et al., *Virology*, 206:935-944 (1995)), pBsTAT, and pCMVsREV, along with a plasmid to supply the envelope in trans, into 293T cells, a cell line with extremely high transfectability. After 48 hours, the supernatant, containing HIV-1 particles with genomes derived from pNL4-3Luc r'e' and gp120-gp41 from the envelope plasmid, was harvested, sterile

filtered, and added to GHOST cells plated on 12-well plates (1-2 x 10^5/well). The GHOST cells have been transduced with a construct containing a humanized, S65T mutant of the green fluorescent protein (GFP), under the inducible control of the HIV-2_{ROD} LTR enhancer-promoter. In these cells the very low basal expression of GFP is induced many-fold upon infection with HIV-1 or HIV-2. Three to four days after infection, the cells were examined using an Olympus IX70 inverted microscope (New Hyde Park, NY) with a 20x objective and a special GFP filter cube (exciter: HQ510/10X, dichroic mirror: Q520lp, emitter: HQ535/20M) (Chroma technology, Brattleboro, VT). Infectivity was calculated as: % Infectivity = 100 x [(number of clusters of GFP-positive cells x average number of cells per GFP-positive cluster) / total number of cells observed]. All infection assays used the following internal controls: mock infection, viral particles without env, viral particles with non-fusogenic env containing the V2E mutation (Freed et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89:70-74 (1992)), and as a positive control, viral particles containing envelope glycoproteins from VSV and A-MLV.

HIV-1 envelope glycoprotein-mediated cell-cell fusion: Target cells were labeled with the cytoplasmic dye 5- and 6-[[(4-chloromethyl)benzoyl]-amino]tetramethylrhodamine (CMTMR) at a concentration of 10 M for 1 hour at 37 °C. When GSL was added to the cells, labeling was performed before addition of GSL to the cell surface. Envelope-expressing cells were labeled with calcein AM at a concentration of 5 M for 1 hour at 37 °C. Calcein-labeled effector cells were cocultured with CMTMR-labeled target cells for 2 hours at 37 °C, and dye redistribution was monitored microscopically as described previously. (Puri et al., *Proc. Natl. Acad. Sci. U.S.A.*, 95:14435-14440 (1998)). The extent of fusion was calculated as: Percent Fusion = 100 x number of bound cells positive for both dyes / number of bound cells positive for CMTMR. When fusion assays were performed on PPMP-treated cells, all media contained PPMP.

Extraction and analysis of cellular GSLs: Total GSLs were extracted from cultured cells as described by Bligh and Dyer. (Bligh and Dyer, *Canadian J. Biochem. Physiol.*, 37:911-917 (1959)). Briefly, 10^7 Ghost 345 cells, suspended with trypsin-EDTA in PBS from Gibco/BRL (Gaithersburg, MD), were pelleted at 450 x g for 5 minutes. The cell pellet was resuspended in 0.5ml H_2O, which was added to 2ml $CHCl_3$:MeOH (2:1, v/v). After vortexing, 0.5ml $CHCl_3$ and 0.5ml H_2O were added, and the suspension was vortexed and centrifuged at 100 x g for 5 minutes to separate the two phases. The extract in the lower phase was then removed for storage, and the $CHCl_3$-H_2O step was repeated twice with the aqueous phase. Extracted GSL were pooled, dried under N_2, resuspended in 100 l $CHCl_3$:MeOH (2:1, v/v) and stored at -20 C until use. The total GSL composition of cells before and after treatment with PPMP was analyzed by thin layer chromatography (TLC) developed in $CHCl_3$:MeOH:10%KCl_{aq} (50:40:10, v/v). At the end of the run, the plate was air dried, sprayed with Resorcinol (Kundu, Methods Enzymol., 72:185-204 (1981)), heated at 100 °C for 20 minutes to develop the spots, and scanned with a Fluor-S Multilmager (Bio-Rad, Hercules, CA).

Flow Cytometry: Ghost 345 cells, harvested with trypsin-EDTA in PBS from Gibco/BRL (Gaithersburg, MD), were centrifuged at 450 x g and resuspended at 10^5 cells/ml in PBS with 5%FBS and 5% normal mouse serum (NMS). After incubating for 15 minutes at room temperature, cells were washed twice in PBS with 0.1% BSA and resuspended at 10^7 cells/ml (in 100ul) in PBS with 5%FBS and 5% NMS. Either PE-conjugated mouse IgG anti-CXCR4 (12G5) (1:5) from Pharmingen (San Diego, CA), PE-conjugated mouse IgG anti-CD4 (OKT4) (1:10) from Ortho Diagnostics (Raritan, NJ), or mouse IgG anti-CCR5 (2D7) (1:400), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, was added to each sample. Cells were incubated at 4 °C for 1 hour and washed twice in PBS with 0.1% BSA. Anti-CCR5 labeled cells were then resuspended in 100 l of PBS with 5%FBS and 5% NMS, incubated for 1 hour at 4 °Cwith FITC-conjugated goat IgG anti-mouse antibody (1:50) from Sigma (St. Louis, MO), and washed as above. Samples were fixed in PBS with 1% paraformaldehyde and resuspended in 1ml of PBS to be read by a FACScalibur (Becton Dickinson, San Jose, CA) at 10,000 events/sample. Unlabeled cells and cells incubated without the primary antibody were used as controls.

CD4-chemokine receptor association: Immunoprecipitation was done according to a previously reported procedure (Xiao et al., *Proc. Natl. Acad. Sci. U.S.A.*, 96:7496-7501 (1999)) with some modifications. Briefly, cells were collected and washed with PBS once. Cells were suspended in ice-cold PBS at a final density of $5x10^6$ /ml. One ml of the cell suspension was used for one immunoprecipitation sample. Antibodies were added to the cell suspension at a final concentration of 3 g/ml, and incubated with gentle mixing for 4 hours at 4°C. Cells were then collected by centrifugation and lysed using a lysis buffer. (Xiao et al., *Proc. Natl. Acad. Sci. U.S.A.*, 96:7496-7501 (1999)). After 40 min of incubation with gentle mixing, the supernatant was obtained by centrifugation at top speed for 25 min in a refrigerated Eppendorf centrifuge. Ten l of protein G-sepharose beads (Sigma, St. Louis, MO) prewashed with PBS was added to each sample and then incubated at 4°C for 14 hours. Protein G-sepharose beads were then washed four times, each with 1ml of ice cold lysis buffer. Samples were then eluted by adding 4X sample buffer for SDS-PAGE gel and boiled for 5 min. Samples were run on a 10% SDS-PAGE gel and Western blot was performed using the Supersignal Chemiluminescent Substrate from Pierce (Rockford, Il).

Chemotaxis Assay: The migration of GHOSTX4 cells was assessed by a 48-well microchamber technique. (Ben-Baruch et al., *J. Biol. Chem.*, 270:22123-22128 (1995)). Different concentrations of SDF-1 (Peprotech, NJ) were placed in the lower wells of the chamber. The Ghost/CXCR4 cells (50 l, 10^6/ml) were loaded in the upper wells. The lower and upper wells were separated by a polycarbonate filter (PVPF, 10 m pore-size; Poretics, CA) precoated with 50 g/ml collagen type 1 for 2 h at 37 °C. The chamber was incubated at 37 °C for 5 h in humidified air with 5% CO_2. At the end of the incubation, after removal of non-migrating cells, the filter was fixed and stained with Diff-Quik (Biochemical Sciences, NJ). Using three high power fields under light microscopy, the cells migrating across the filter were counted in triplicate with all samples coded. The chemotaxis index was calculated as: CI = Number of cells

migrating to chemokines/Number of cells migrating to medium. The significance of the difference between test and control groups was analyzed by paired Student's t test.

Measurement of CD4 and CXCR4-induced conformational changes: Fluorescence changes of the hydrophobicity-sensitive dye, bis-ANS, resulting from conformational changes in gp120-gp41 were monitored according to the procedure described previously (Jones et al., *J. Biol. Chem.*, 273:404-409 (1998)) with some modifications. Untreated and PPMP-treated HeLaCD4 cells were plated on 35 mm dishes with coverslip cutouts in the center. TF228 cells, which constitutively express the X4-utilizing (IIIB) gp120-gp41, were labeled with calcein as described above and then added to the culture dish. Bis-ANS was added at 2 g/ml to culture medium without serum. Once the TF228 cells were touching the target cells as determined by brightfield and calcein fluorescence, images were recorded using the quantitative light microscopy set-up described previously. (Jones et al., *J. Biol. Chem.*, 273:404-409 (1998). The fluorescence intensity averaged from regions of interest (ROIs) drawn around individual gp120-gp41-expressing cells was monitored at 37 C as a function of time following contact of effector and target cells.

Addition of GSL to CD4⁺ Cells: The addition of GSL to the plasma membrane of cells was performed as described previously. (Puri et al., *Proc. Natl. Acad. Sci.* U.S.A., 95:14435-14440 (1998)). Briefly, liposomes containing Egg PC:Egg PE: GSL (3:1.5:1, wt/wt) were prepared in PBS (Ca/Mg free) by extrusion through a 0.2 mm filter using an extruder from Lipex Biomembranes (Vancouver, BC) to a final lipid concentration of 0.9 mg/ml total lipid. Target cells plated on 35 mm dishes with coverslip cutouts in the center (at 5 x 10^4/dish) were infected with the recombinant viral strain X-31 (H3N2) of influenza virus[34] overnight at 37 °C. Target cells were treated with 5 g/ml trypsin for 5 min at room temperature to activate the HA on the cell surface. Liposomes were allowed to bind to the HA-expressing target cells for 30 min at room temperature. Liposome-cell fusion was induced by 60 sec exposure of the cells to pH 5.2, followed by incubation in D10 at pH 7.4. The modified cells were then used as targets in the cell-cell fusion assays described above.

Although the invention has been described with reference to embodiments and examples, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All references cited herein are hereby expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of enhancing fusion of a virus with a cell comprising:

providing globotriaosylceramide to the cell such that the globotriaosylceramide is incorporated in the plasma membrane of the cell; and

infecting the cell with the virus.

2. The method of Claim 1, wherein the virus comprises nucleotide sequence encoding HIV proteins.

3. The method of Claim 1, wherein the cell expresses CD4.

4. A method of enhancing fusion of a virus with a cell comprising:

inducing a cell to produce globotriaosylceramide; and

infecting the cell with a virus.

5. The method of Claim 4, wherein the virus comprises nucleotide sequence encoding HIV proteins.

6. The method of Claim 4, wherein the cell expresses CD4.

7. A method of enhancing fusion of two cells comprising:

providing a first cell;

treating the first cell with globotriaosylceramide such that the globotriaosylceramide is incorporated in the plasma membrane of the cell;

providing a second cell; and

contacting the first cell with the second cell.

8. The method of Claim 7, wherein at least one cell expresses CD4.

9. The method of Claim 7, wherein at least one cell has gp120 on its plasma membrane.

10. A cell comprising an enhanced ability to fuse with a member of the group consisting of a virus, a second cell or a liposome wherein the member of the group comprises gp120 and the cell has been manipulated to have sufficient globotriaosylceramide on its cell surface to mediate enhanced fusion.

11. A method of enhancing delivery of an agent to a cell comprising:

providing a liposome that contains an agent to be delivered to the cell, wherein the liposome has gp120 on its plasma membrane; and

contacting the liposome with a cell that has globotriaosylceramide.

12. A method of prevention and treatment of HIV infection and AIDS comprising administering an agent that inhibits the synthesis of glycosphingolipids.

13. Use of an agent that inhibits the synthesis of glycosphingolipids for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

14. A method of prevention and treatment of HIV infection and AIDS comprising administering an agent that inhibits the synthesis of a glucosyl ceramide.

15. Use of an agent that inhibits the synthesis of glucosyl ceramide for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

16. A method of prevention and treatment of HIV infection and AIDS comprising administering an agent that inhibits the synthesis of globotriaosylceramide.

17. Use of an agent that inhibits the synthesis of globotriaosylceramide for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

18. A method of prevention and treatment of HIV infection and AIDS comprising administering a member of the group consisting of NB-DGJ, an NB-DGJ analog, PPMP, a PPMP analog, PPPP and a PPPP analog.

19. Use of a member of the group consisting of NB-DGJ, an NB-DGJ analog, PPMP, a PPMP analog, PPPP and a PPPP analog for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

20. A method of prevention and treatment of HIV infection and AIDS comprising administering an antisense oligonucleotide or ribozyme that inhibits an enzyme involved in glycosphingolipid synthesis.

21. Use of an antisense oligonucleotide or ribozyme that inhibits an enzyme involved in glycosphingolipid synthesis for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

22. The method according to Claim 20 or the use according to Claim 21, wherein the antisense oligonucleotide or ribozyme inhibits the synthesis of a glucosyl ceramide.

23. The method according to Claim 20 or the use according to Claim 21, wherein the antisense oligonucleotide or ribozyme inhibits the synthesis of globotriaosylceramide.

24. A method of prevention and treatment of HIV infection and AIDS comprising administering a monoclonal antibody that inhibits an enzyme involved in glycosphingolipid synthesis.

25. Use of a monoclonal antibody that inhibits an enzyme involved in glycosphingolipid synthesis for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

26. The method according to Claim 24 or the use according to Claim 25, wherein the monoclonal antibody inhibits the synthesis of a glucosyl ceramide.

27. The method according to Claim 24 or the use according to Claim 25, wherein the monoclonal antibody inhibits the synthesis of globotriaosylceramide.

28. A method of prevention and treatment of HIV infection and AIDS comprising administration of an agent that cleaves a sugar residue on a glycosphingolipid.

29. Use of an agent that cleaves a sugar residue on a glycosphingolipid for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

30. The method according to Claim 28 or the use according to Claim 29, wherein the agent that cleaves the sugar residue comprises a glycosphingolipidase.

31. A method of prevention and treatment of HIV infection and AIDS comprising administration of an agent that cleaves a sugar residue on a glucosyl ceramide.

32. Use of an agent that cleaves a sugar residue on a glucosyl ceramide for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

33. The method according to Claim 31 or the use according to Claim 32, wherein the agent that cleaves the sugar residue comprises a glucosyl ceramidase.

34. A method of prevention and treatment of HIV infection and AIDS comprising administration of an agent that cleaves a sugar residue on a globotriaosylceramide.

35. Use of an agent that cleaves a sugar residue on a globotriaosylceramide for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

36. The method according to Claim 34 or the use according to Claim 35, wherein the agent that cleaves the sugar residue comprises a globotriaosylceramidase.

37. A method of prevention and treatment of HIV infection and AIDS comprising administration of an agent that binds to a glycosphingolipid.

38. Use of an agent that binds to a glycosphingolipid for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

39. The use of Claim 38, wherein the agent that binds to the glycosphingolipid comprises a member of the group consisting of a monoclonal antibody, a recombinant protein, a synthetic peptide, a toxin, a lectin and a chemical.

40. The use of Claim 39, wherein the toxin comprises a verotoxin or protein homologous to a verotoxin.

41. A method of prevention and treatment of HIV infection and AIDS comprising administration of an agent that binds to a glucosyl ceramide.

42. Use of an agent that binds to a glucosyl ceramide for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

43. The use of Claim 42, wherein the agent that binds to the glucosyl ceramide comprises a member of the group consisting of a monoclonal antibody, a recombinant protein, a synthetic peptide, a toxin, a lectin and a chemical.

44. The use of Claim 43, wherein the toxin comprises a verotoxin or protein homologous to a verotoxin.

45. A method of prevention and treatment of HIV infection and AIDS comprising administration of an agent that binds to globotriaosylceramide.

46. Use of an agent that binds to a globotriaosylceramide for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

47. The use of Claim 46, wherein the agent that binds to the globotriaosylceramide comprises a member of the group consisting of a monoclonal antibody, a recombinant protein, a synthetic peptide, a toxin, a lectin and a chemical.

48. The use of Claim 47, wherein the toxin comprises a verotoxin or protein homologous to a verotoxin.

49. A method of prevention and treatment of HIV infection and AIDS comprising administering a glycosphingolipid to a patient suffering from HIV infection and/or AIDS.

50. Use of a glycosphingolipid for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

51. The use according to Claim 50, wherein the glycosphingolipid is a glucosyl ceramide.

52. The use according to Claim 51, wherein the glucosyl ceramide is a globotriaosylceramide.

53. The use according to Claim 52, wherein the globotriaosylceramide is administered in a multimeric form.

54. The use according to Claim 53, wherein the globotriaosylceramide is attached to a support.

55. A method of isolating HIV comprising:

providing a support having immobilized globotriaosylceramide;

applying a solution having HIV to the support;

allowing the HIV in the solution to bind to the support for a sufficient time;

washing away any unbound material from the support; and

eluting the bound HIV from the support.

56. The method of Claim 55, wherein the support is a composite support.

57. A composite support comprising a material to which is attached Gb3 and at least one member of the group consisting of CD4 and a chemokine receptor.

58. A method of removing HIV from a contaminated solution comprising:

providing a support having immobilized globotriaosylceramide;

applying a solution of contaminated HIV to the support; and

allowing the HIV in the solution to bind to the support for a sufficient time.

59. The method of Claim 58, wherein the contaminated solution is of human origin.

60. A method of removing HIV from a human comprising:

providing a dialysis machine having a support of immobilized globotriaosylceramide;

transferring blood from the human to the dialysis machine; and

allowing the blood to circulate through the machine for a time sufficient to allow binding of HIV to the immobilized globotriaosylceramide support.

61. Use of a dialysis machine comprising a glycosphingolipid for the manufacture of a medicament for the prevention and treatment of HIV infection and AIDS.

62. A dialysis filter comprising at least one layer of globotriaosylceramide.

63. The filter of Claim 62, wherein the layer of globotriaosylceramide further comprises at least one member of the group consisting of CD4 and chemokine receptors.

64. A method of screening for a prophylactic agent that prevents HIV infection or AIDS comprising:

providing gp 120 immobilized to a substrate;

providing the prophylactic agent to the immobilized gp120;

providing globotriaosylceramide to the prophylactic agent and immobilized gp 120; and

determining the amount of globotriaosylceramide bound to the immobilized gp 120.

65. A method of screening for a prophylactic agent that prevents HIV infection or AIDS comprising:

providing globotriaosylceramide immobilized to a support;

providing the prophylactic agent to the immobilized globotriaosylceramide;

providing gp 120 to the prophylactic agent and immobilized globotriaosylceramide; and

determining the amount of gp 120 bound to the immobilized globotriaosylceramide.

66. A method of screening for a therapeutic agent that prevents or treats HIV infection or Aids comprising:

providing gp 120 immobilized to a substrate;

providing globotriaosylceramide to the immobilized gp 120;

providing the therapeutic agent to the immobilized gp120 and globotriaosylceramide; and

determining the amount of globotriaosylceramide that remains bound to the immobilized gp 120.

67. The method according to Claim 66, wherein the therapeutic agent is added simultaneously with the addition of globotriaosylceramide.

68. The method according to Claim 66, wherein the therapeutic agent is added after the addition of globotriaosylceramide.

69. A method of screening for a therapeutic agent that prevents or treats HIV infection or AIDS comprising:

providing globotriaosylceramide immobilized to a substrate;

providing gp 120 to the immobilized globotriaosylceramide;

providing the therapeutic agent to the immobilized globotriaosylceramide; and

determining the amount of gp 120 bound to the immobilized globotriaosylceramide.

70. The method according to Claim 69, wherein the therapeutic agent is added simultaneously with the addition of gp 120.

71. The method according to Claim 69, wherein the therapeutic agent is added after the addition of gp120.

72. A diagnostic tool comprising globotriaosylceramide attached to a support.

73. The diagnostic tool of Claim 72, wherein the support further comprises at least one member of the group consisting of CD4 and chemokine receptors.

74. A method of detecting HIV comprising:

providing a support to which globotriaosylceramide is attached;

contacting the support with a fluid from an HIV infected subject;

washing the unbound material from the support; and

detecting the bound HIV virus.

75. A method of screening an agent that enhances cell fusion comprising:

providing a cell having CD4;

treating the cell having CD4 with the agent that enhances cell fusion;

contacting the treated cell having CD4 with a member of the group consisting of a liposome, a second cell and a virus, wherein the member of the group comprises gp120; and

determining whether the treated cell having CD4 has fused with the member of the second group.

76. The method of Claim 75, wherein the member of the group is treated with the agent that enhances cell fusion.

77. A method of screening an agent that prevents or inhibits fusion comprising:

providing a cell having CD4;

treating the cell having CD4 with the agent that prevents or inhibits fusion;

contacting the treated cell having CD4 with a member of the group consisting of a liposome, a second cell and a virus, wherein the member of the group comprises gp120; and

determining whether the treated cell having CD4 has fused with the member of the second group.

78. The method of Claim 77, wherein the member of the group is treated with the agent that prevents or inhibits fusion.

79. A method of screening a fusion enhancing agent comprising:

providing gp 120 immobilized to a substrate;

providing globotriaosylceramide to the immobilized gp 120;

providing the fusion enhancing agent to the immobilized gp120 and globotriaosylceramide; and

determining the amount of globotriaosylceramide that remains bound to the immobilized gp 120.

80. The method according to Claim 79, wherein the fusion enhancing agent is added simultaneously with the addition of globotriaosylceramide.

81. The method according to Claim 79, wherein the fusion enhancing agent is added after the addition of globotriaosylceramide.

82. A method of screening a fusion enhancing agent comprising:

providing globotriaosylceramide immobilized to a substrate;

providing gp 120 to the immobilized globotriaosylceramide;

providing the fusion enhancing agent to the immobilized globotriaosylceramide; and

determining the amount of gp 120 bound to the immobilized globotriaosylceramide.

83. The method according to Claim 82, wherein the fusion enhancing agent is added simultaneously with the addition of gp 120.

84. The method according to Claim 82, wherein the fusion enhancing agent is added after the addition of gp120.

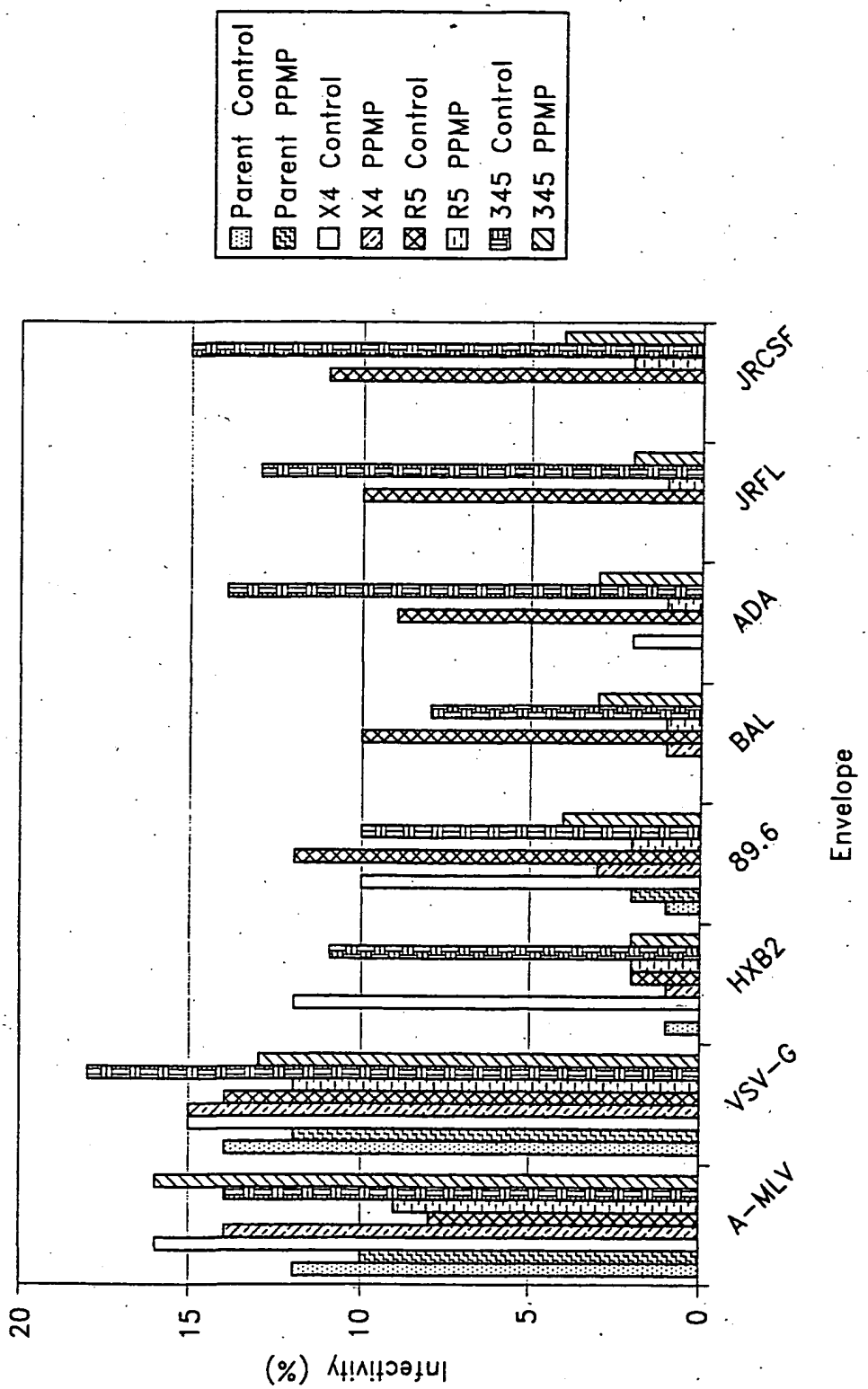

Parent Control
Parent PPMP
X4 Control
X4 PPMP
R5 Control
R5 PPMP
345 Control
345 PPMP
JRCSF
JRFL
ADA
BAL
89.6
HXB2
VSV-G
A-MLV
Envelope
Infectivity (%)
0
5
10
15
20

FIG. 1

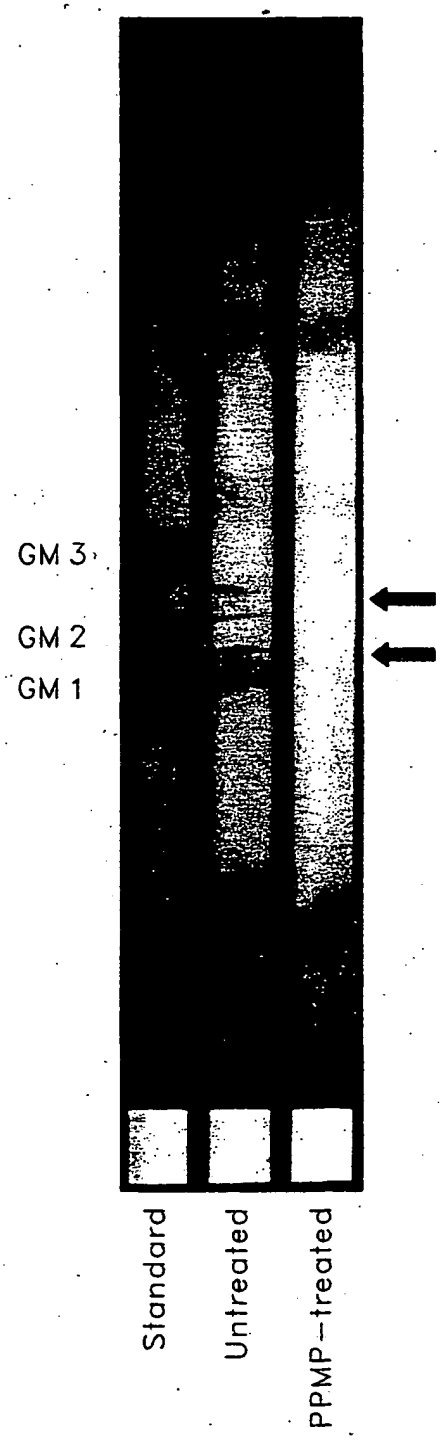


FIG. 2

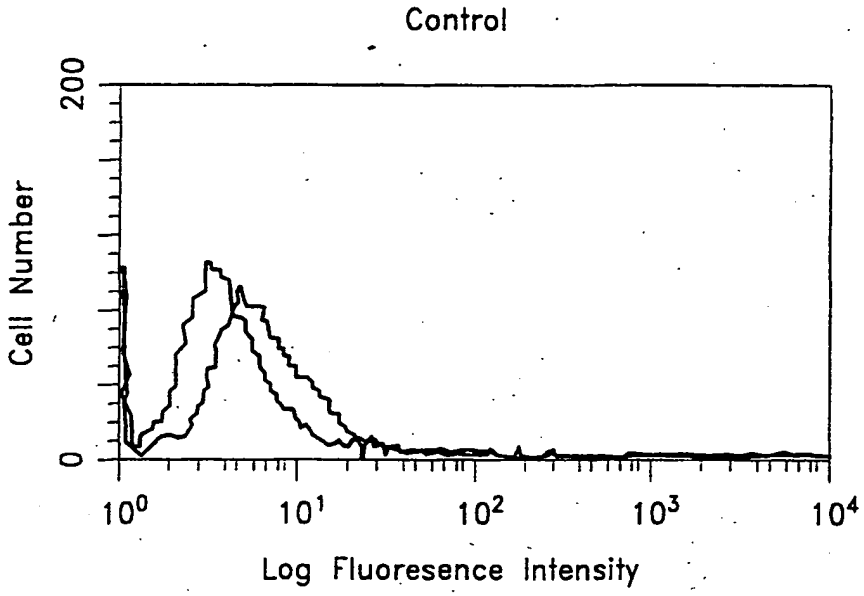


FIG. 3A-1

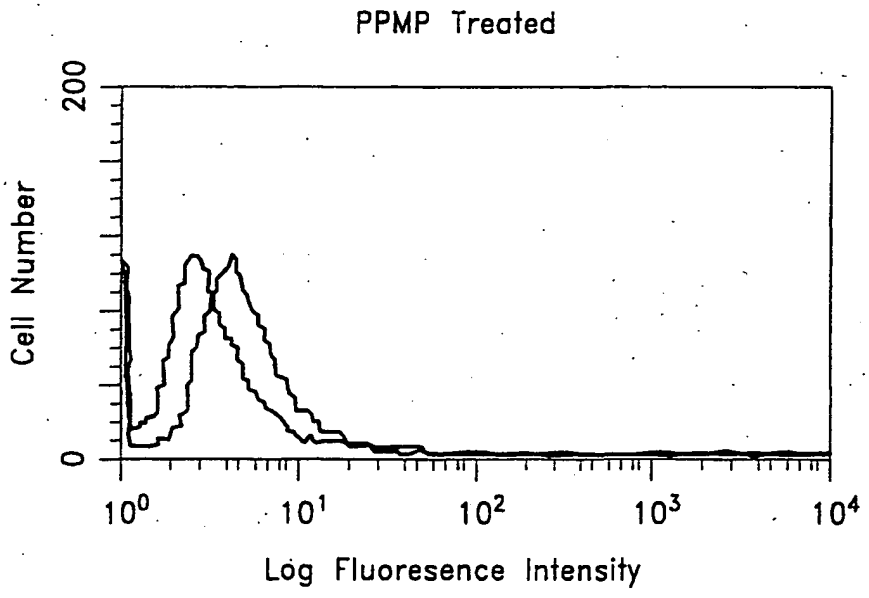


FIG. 3A-2

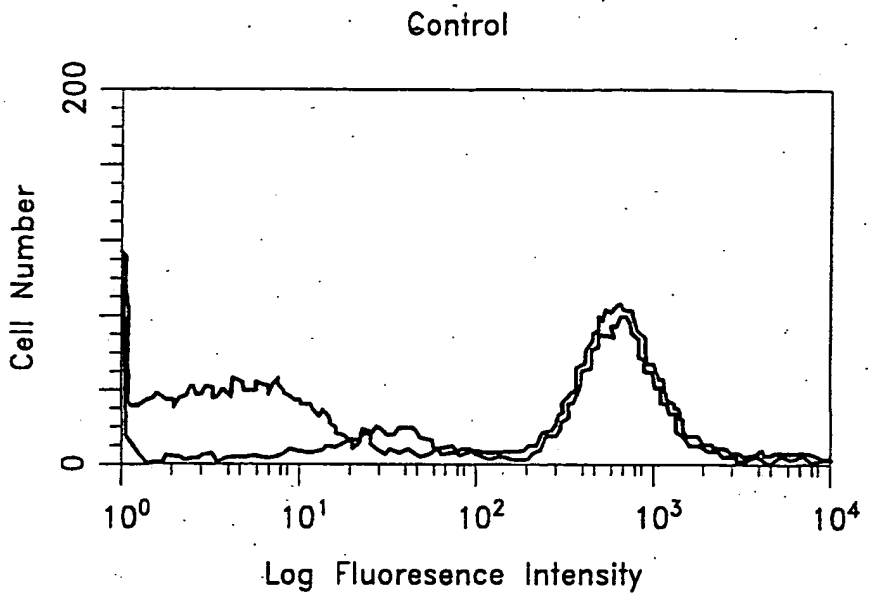


FIG. 3B-1

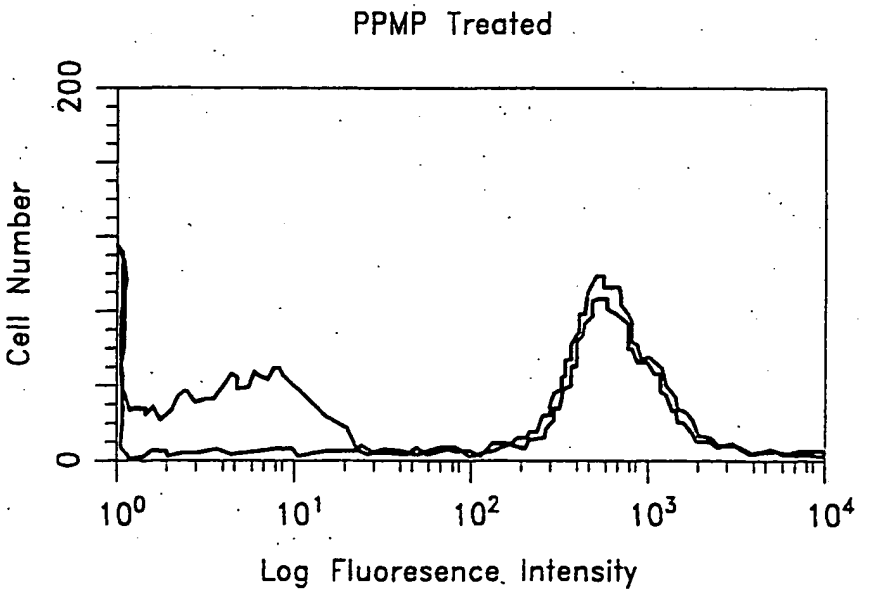


FIG. 3B-2

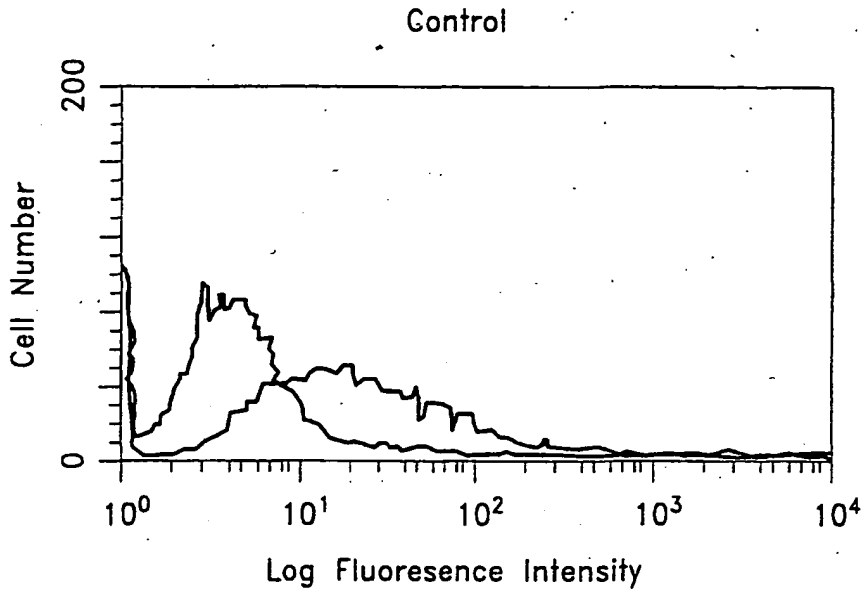


FIG. 3C-1

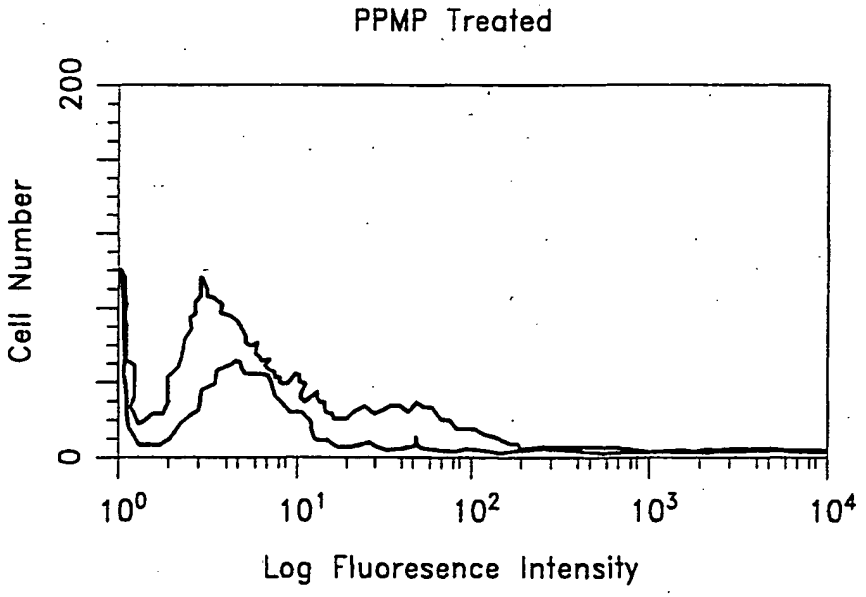


FIG. 3C-2

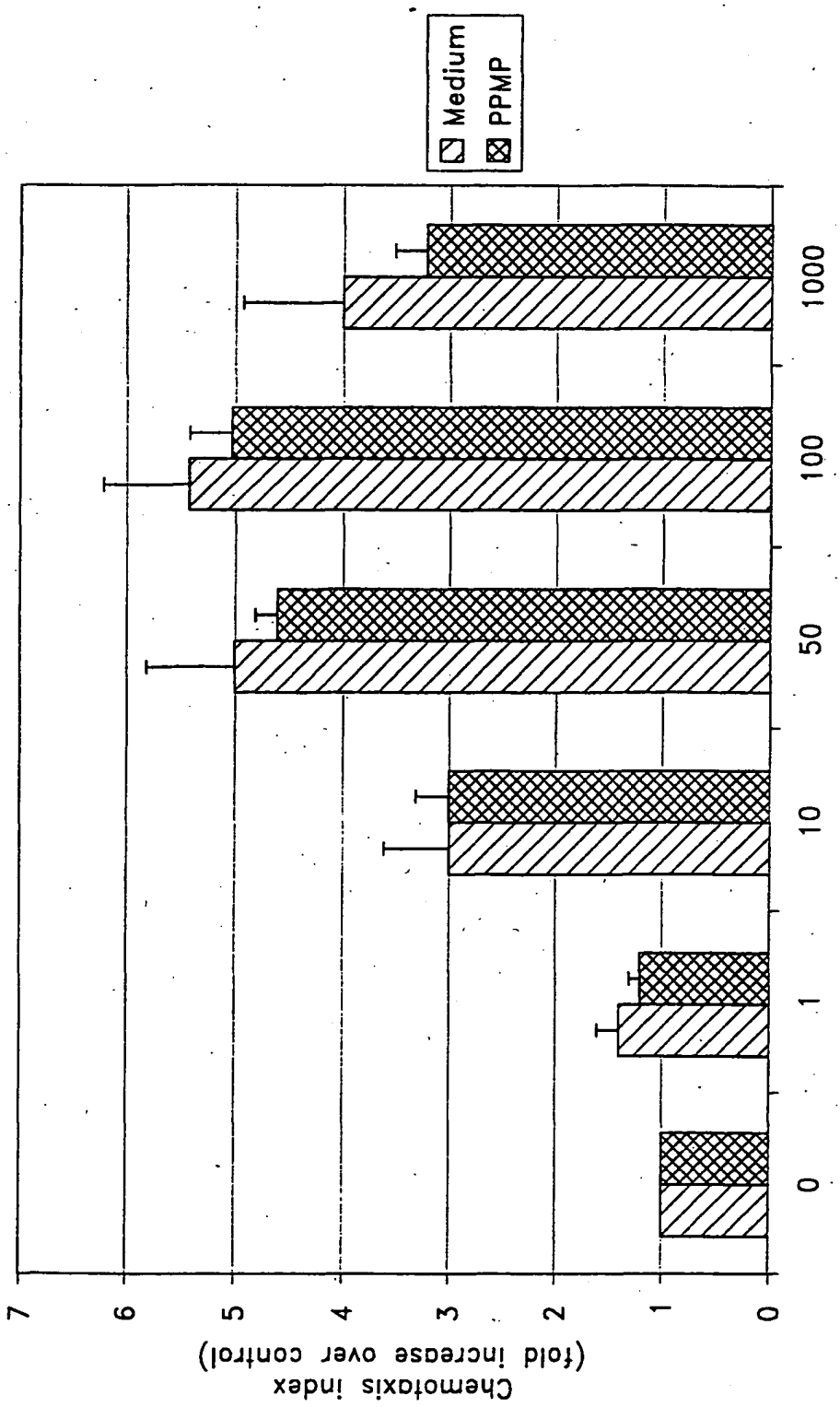

Medium
PPMP
Chemotaxis index
(fold increase over control)
0
1
2
3
4
5
6
7
0
1
10
50
100
1000
SDF-1α(ng/ml)

FIG. 4

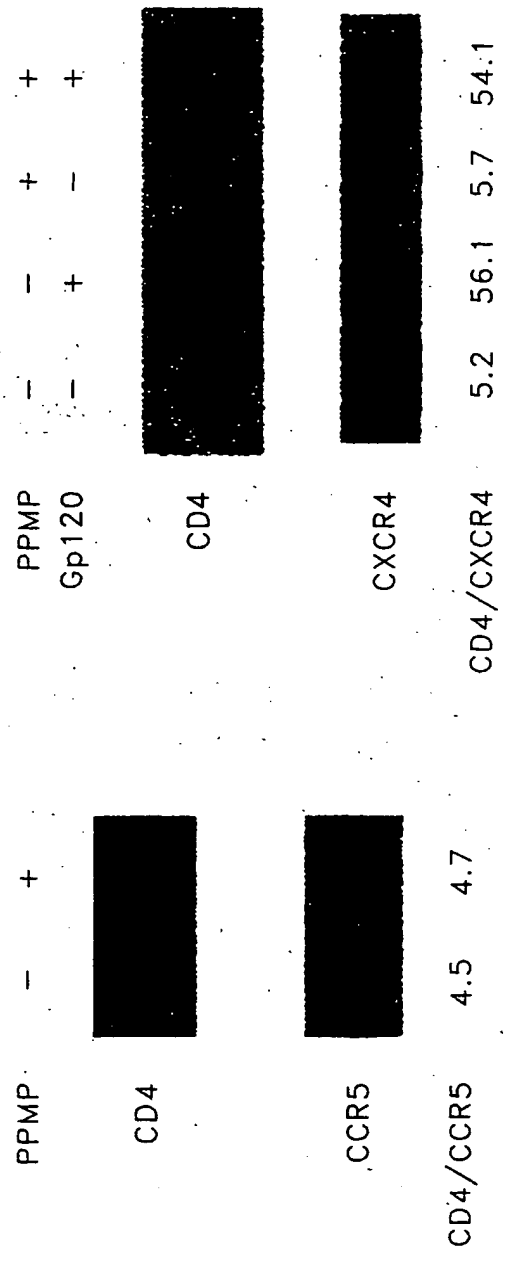

PPMP
Gp120
CD4
CXCR4
CD4/CXCR4
5.2
56.1
5.7
54.1
PPMP
CD4
CCR5
CD4/CCR5
4.5
4.7

FIG. 5B

FIG. 5A

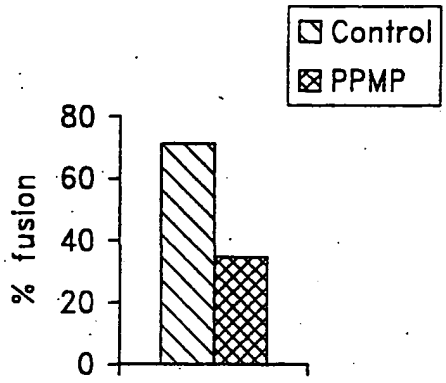

Control
PPMP
% fusion
80
60
40
20
0

FIG. 5C

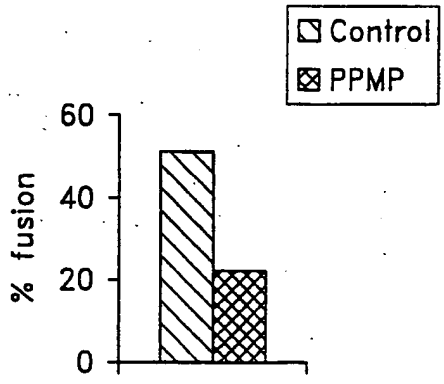

Control
PPMP
% fusion
60
40
20
0

FIG. 5D

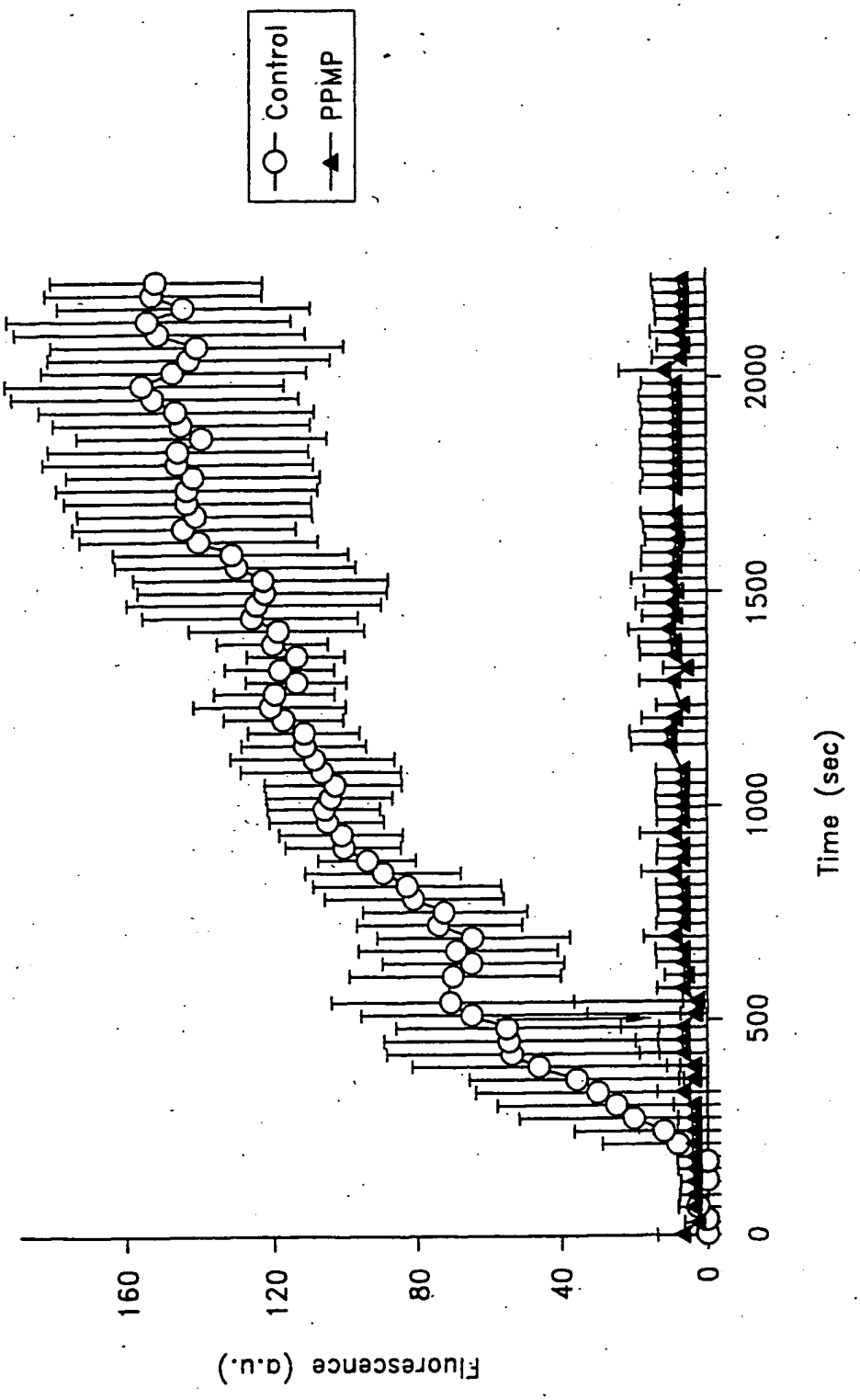

Control
PPMP
Fluorescence (a.u.)
160
120
80
40
0
0
500
1000
1500
2000
Time (sec)

FIG. 6

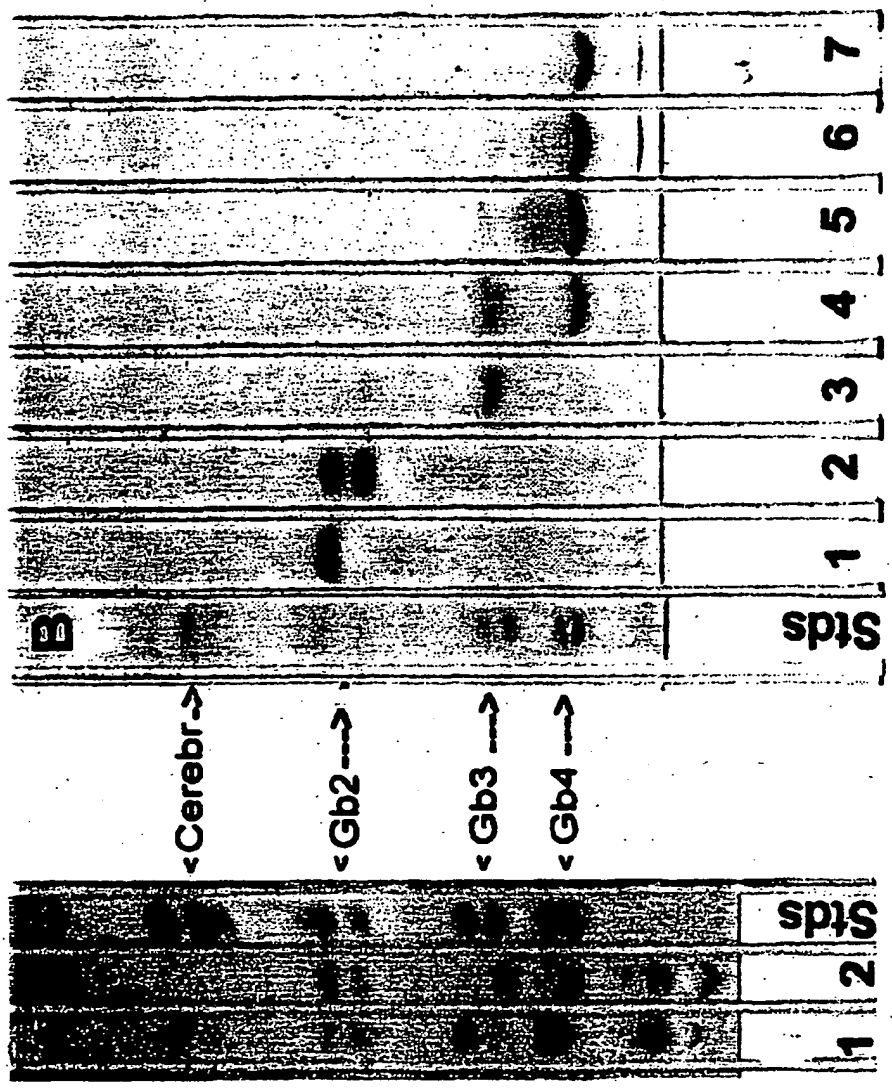


FIG. 7B

FIG. 7A

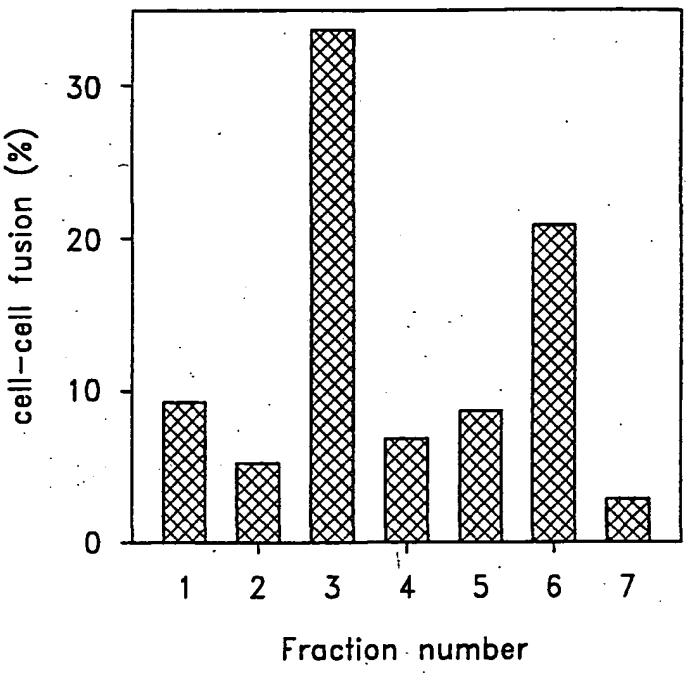


FIG. 7C

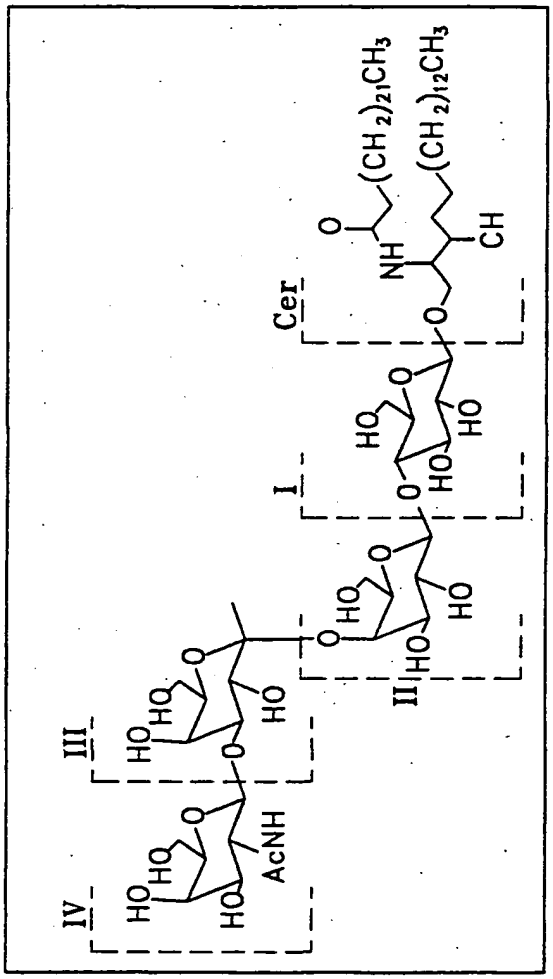


FIG. 8A

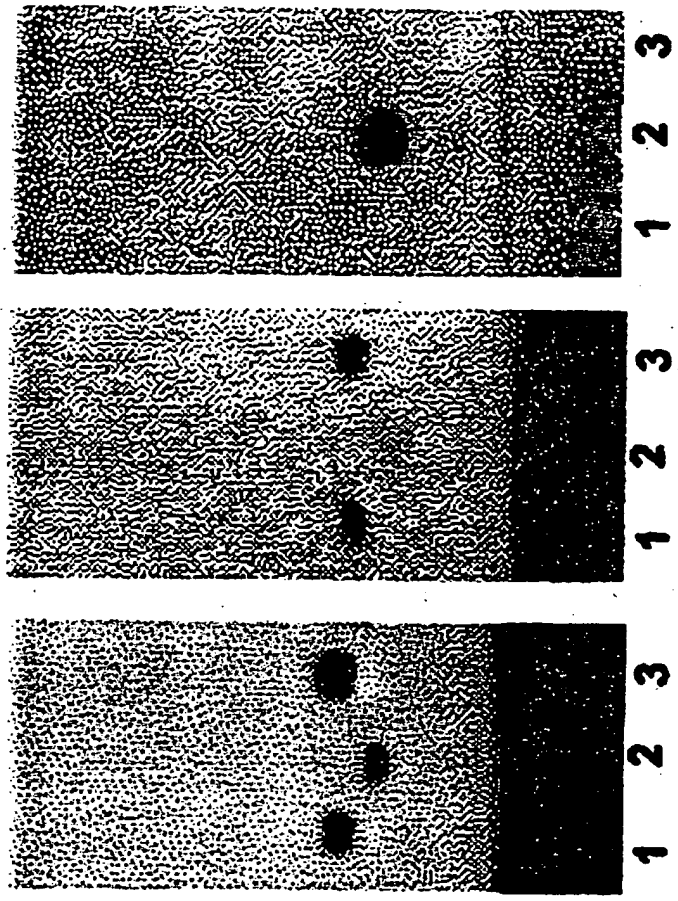


FIG. 8C

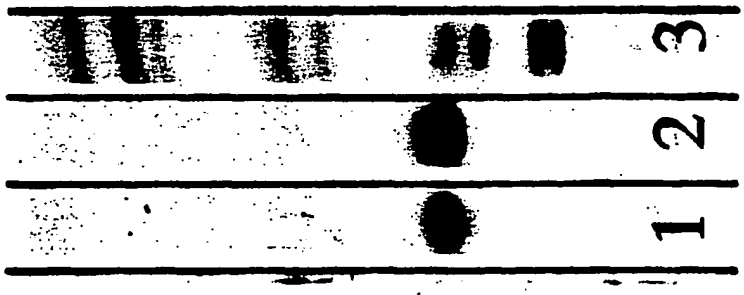


FIG. 8B

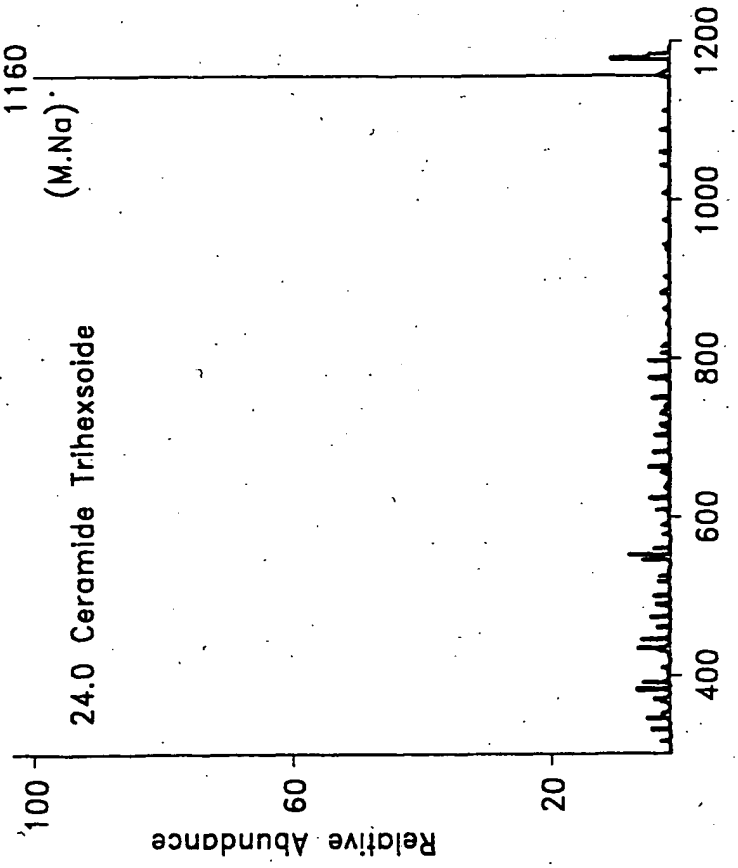

24.0 Ceramide Trihexsoide
1160
(M.Na)
Relative Abundance
100
60
20
400
600
800
1000
1200
N/Z

FIG. 8D

FIG. 8E-1

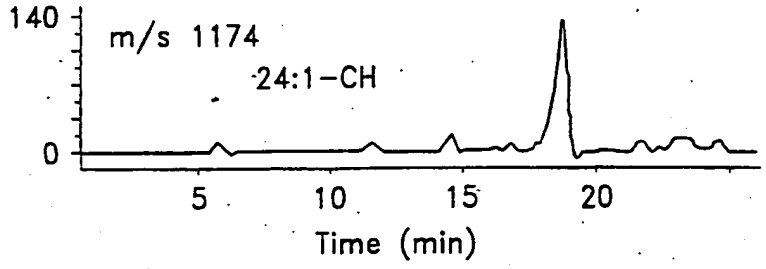


FIG. 8E-2

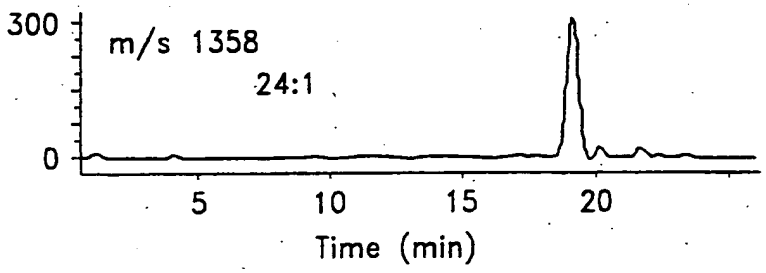


FIG. 8E-3

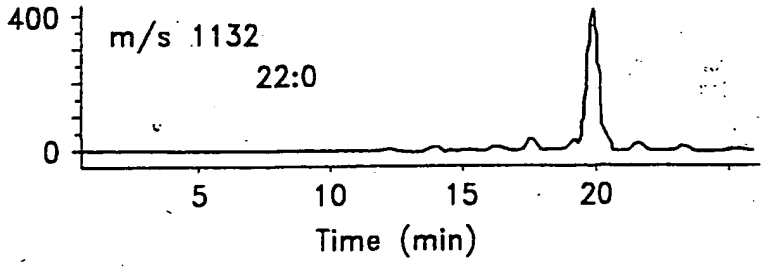


FIG. 8E-4

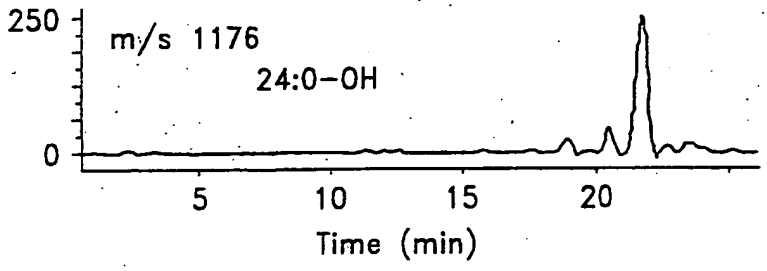
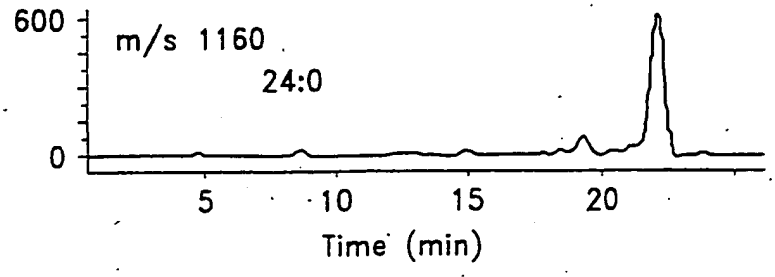


FIG. 8E-5



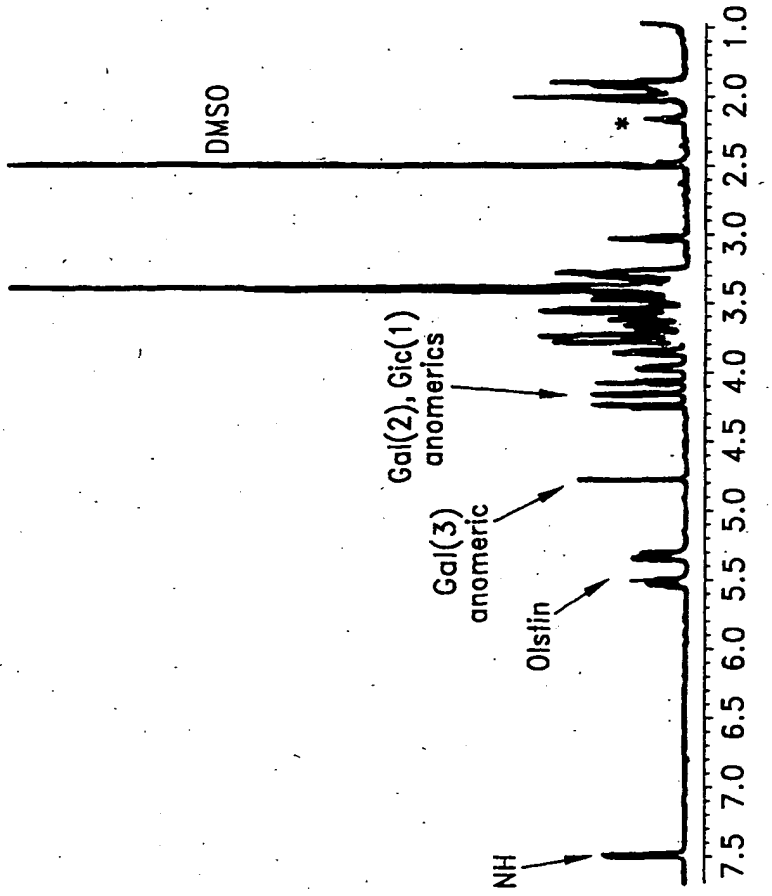

DMSO
*
Gal(2), Glc(1) anomerics
Gal(3) anomeric
Olstin
NH
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.0

FIG. 8F

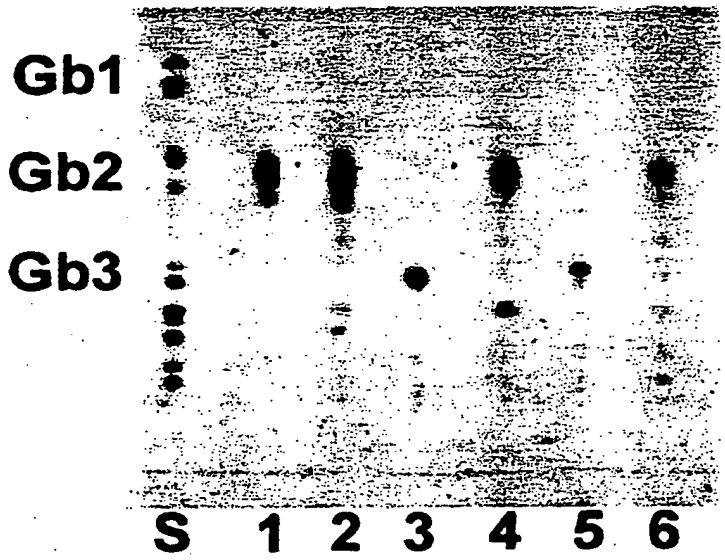


FIG. 9A

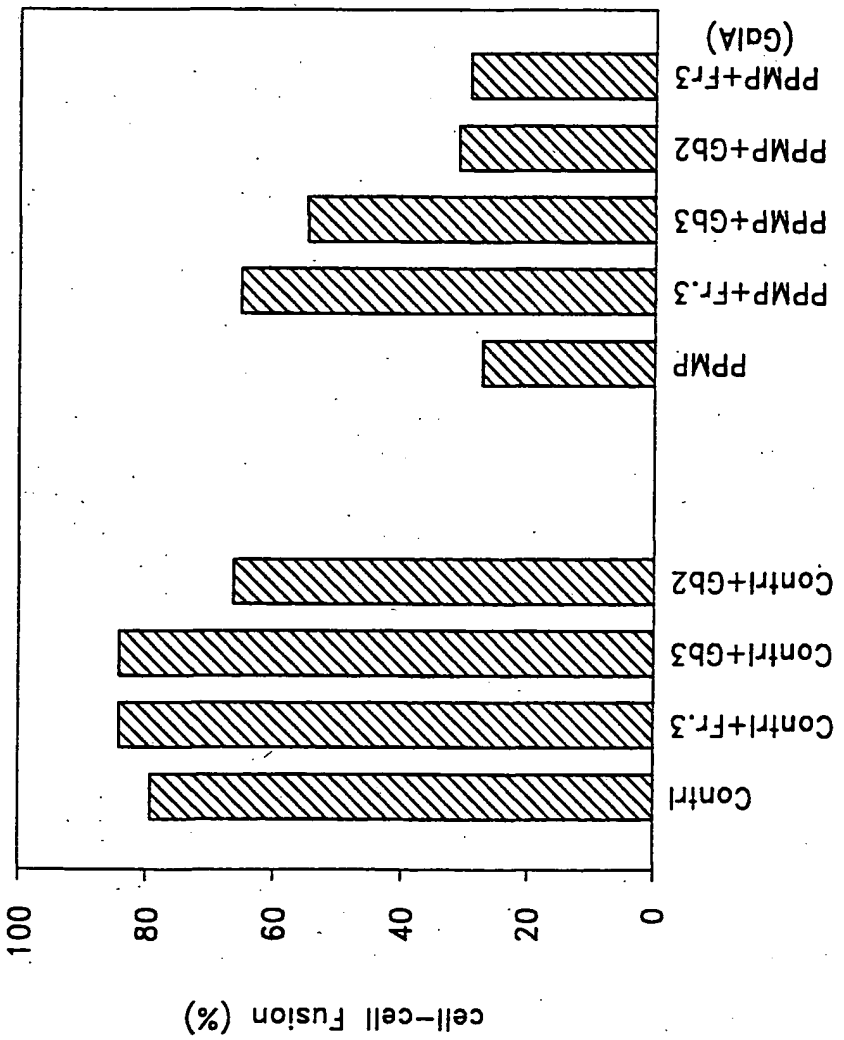

cell–cell Fusion (%)
100
80
60
40
20
0
Contrl
Contrl+Fr.3
Contrl+Gb3
Contrl+Gb2
PPMP
PPMP+Fr.3
PPMP+Gb3
PPMP+Gb2
PPMP+Fr3
(GalA)

FIG. 9B

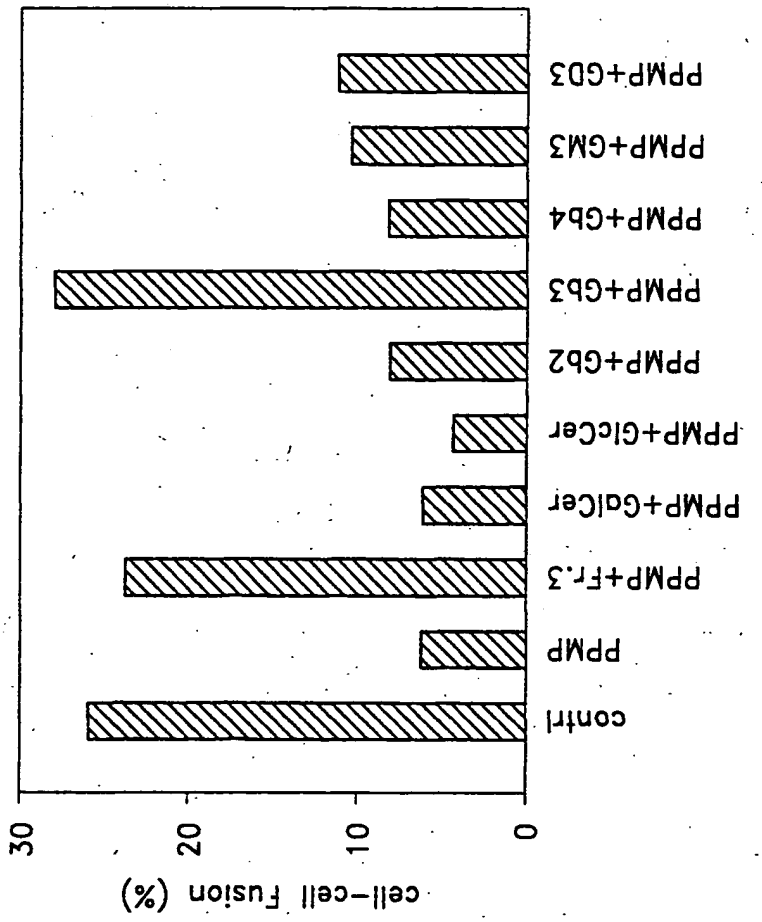

cell–cell Fusion (%)
0
10
20
30
contrl
PPMP
PPMP+Fr.3
PPMP+GalCer
PPMP+GlcCer
PPMP+Gb2
PPMP+Gb3
PPMP+Gb4
PPMP+GM3
PPMP+GD3

FIG. 10

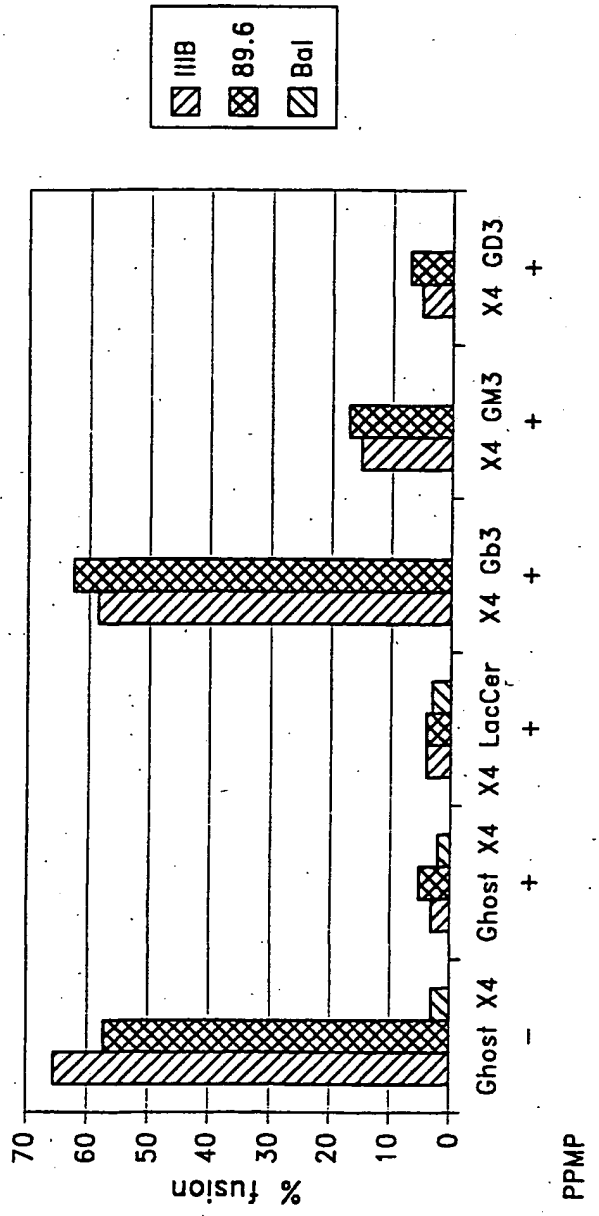

IIIB
89.6
Bal
% fusion
70
60
50
40
30
20
10
0
Ghost X4
Ghost X4
X4 LacCer
X4 Gb3
X4 GM3
X4 GD3
–
+
+
+
+
+
PPMP

FIG. 11A

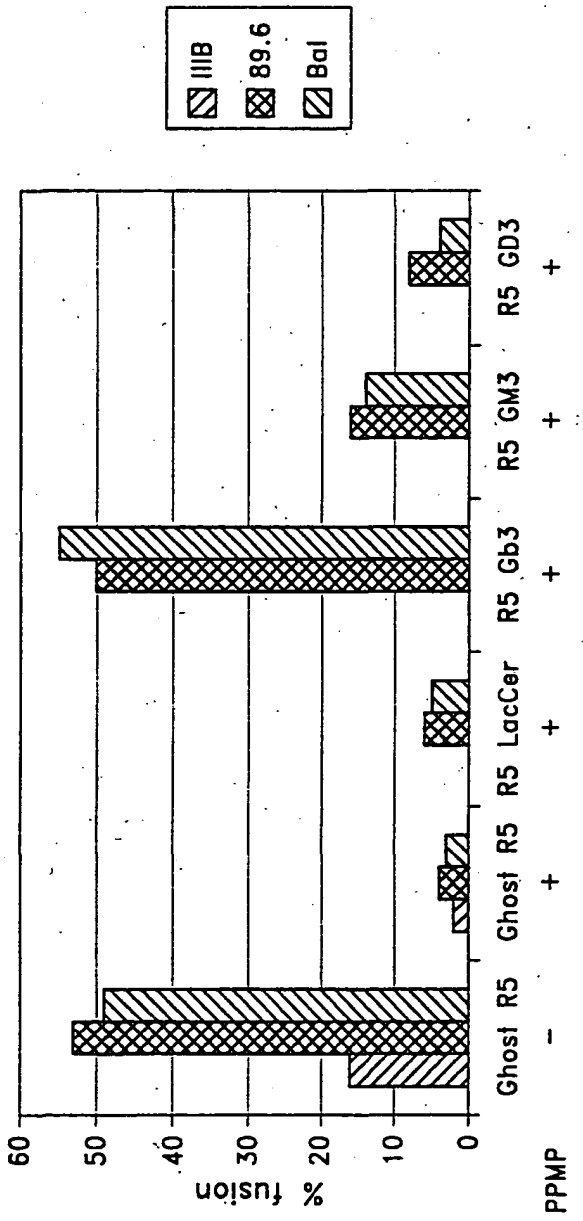

IIIB
89.6
Bal
% fusion
60
50
40
30
20
10
0
PPMP
-
+
+
+
+
+
Ghost R5
Ghost R5
R5 LacCer
R5 Gb3
R5 GM3
R5 GD3

FIG. 11B

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 5/12, 5/06, A61K 31/70, 31/395, C12N 7/00, A61M 1/16, A61K 38/46, 39/395, 38/16, 38/17, 48/00, A61P 31/18

A3

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60/108,903 17 November 1998 (17.11.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/108,903 (CON)
Filed on 17 November 1998 (17.11.98)

(71) Applicant *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; c/o National Institutes of Health, Office Of Technology Transfer, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852-3804 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BLUMENTHAL, Robert [US/US]; 4506 Gretna Street, Bethesda, MD 20814 (US). PURI, Anu [US/US]; 23 Napa Valley Road, Gaithersburg, MD 20878 (US). HUG, Peter [US/US]; 2020 Baltimore Road, Rockville, MD 20851 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson & Bear, LLP, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660-8016 (US).

(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR (Utility model), KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
23 November 2000 (23.11.00)

(54) Title: IDENTIFICATION OF GLYCOSPHINGOLIPIDS THAT PROMOTE HIV-1 ENTRY INTO CELLS

(57) Abstract

The invention is related to the discovery of cofactors that promote CD4-dependent HIV-1 fusion and entry. More specifically, disclosed herein are biotechnological tools, diagnostics, prophylactics, therapeutics and methods of use of the foregoing for the treatment and prevention of HIV-1 infection that exploit interactions between gp120-gp41 and glycosphingolipids.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/27341

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/12 C12N5/06 A61K31/70 A61K31/395 C12N7/00
A61M1/16 A61K38/46 A61K39/395 A61K38/16 A61K38/17
A61K48/00 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PURI A ET AL: "Human erythrocyte glycolipids promote HIV-1 envelope glycoprotein-mediated fusion of CD4+ cells" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATION, vol. 242, 6 January 1998 (1998-01-06), pages 219-225, XP002141781 cited in the application the whole document --- -/--	1-10, 12-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
4 July 2000	20/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stein, A

1

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INTERNATIONAL SEARCH REPORT

Int... ...onal Application No
PCT/US 99/27341

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	US 5 786 368 A (BUTTERS TERRY D ET AL) 28 July 1998 (1998-07-28) the whole document	12-19
Y	KARPAS A ET AL: "Aminosugar derivatives as potential anti-human immunodeficiency virus agents" PROC NATL ACAD SCI USA, vol. 85, December 1988 (1988-12), pages 9229-9233, XP002141782 the whole document	12-19
X	DELÉZAY O ET AL: "SPC3, a V3 Loop-derived synthetic peptide inhibitor of HIV-1 infection, binds to cell surface glycosphingolipids" BIOCHEMISTRY, vol. 35, 1996, pages 15663-15671, XP002141783 cited in the application	37-39, 41-43, 45-47
Y	the whole document, especially table 2	40,44,48
Y	CA 2 163 716 A (LINGWOOD CLIFFORD A ;FARKAS HIMSLEY HANNAH DECEASED (CA); HILL ROB) 25 May 1997 (1997-05-25) the whole document, especially page 7, line 19 - line 23 and claims 1-22	40,44,48
X	WO 89 02918 A (VANDENBARK ARTHUR ALLEN ;OFFNER HALINA (US)) 6 April 1989 (1989-04-06) page 2, line 32 - line 34 page 5, line 1 -page 8, line 27 example 5	49,50
X	US 5 795 869 A (FACCI LAURA ET AL) 18 August 1998 (1998-08-18) the whole document	49,50

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1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/27341

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 47318 A (PROGENICS PHARM INC ;AARON DIAMOND AIDS RESEARCH CE (US)) 18 December 1997 (1997-12-18) page 2, line 1 - line 9 page 3, line 29 -page 5, line 30 page 11, line 1 - line 19 page 15, line 31 -page 16, line 19 claims 27,28 ---	75-78
A	PICARD LAURENT ET AL: "Possible role of nonprotein components of the plasma membrane in CD4-dependent membrane fusion and HIV entry." PERSPECTIVES IN DRUG DISCOVERY AND DESIGN, vol. 5, 1996, pages 143-153, XP000923035 ISSN: 0928-2866 the whole document ---	1-84
A	EP 0 298 280 A (HAPGOOD C V) 11 January 1989 (1989-01-11) page 2, line 10 - line 17 page 4, line 14 - line 44 page 12, line 10 -page 13, line 34 claims 1-36 ---	1-11,74
A	US 5 374 548 A (CARAS INGRID W) 20 December 1994 (1994-12-20) column 4, line 1 - line 8 column 4, line 44 - line 63 column 5, line 25 - line 32 column 10, line 56 - line 65 column 25, line 38 -column 27, line 3 claims 1-18 ---	11
A	ZENG GUICHAO ET AL: "Regulation of glycolipid synthesis in HL-60 cells by antisense oligodeoxynucleotides to glycosyltransferase sequences: Effect on cellular differentiation." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 92, no. 19, 1995, pages 8670-8674, XP002141784 1995 ISSN: 0027-8424 the whole document ---	20-23
A	ZENG G ET AL: "Permanent alteration of endogenous gangliosides in neuroblastoma cells by stable transfection with antisense vector." ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, (1998 JUN 19) 845 431. , XP000915132 the whole document ---	20-23
	-/--	

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INTERNATIONAL SEARCH REPORT

Inte...onal Application No
PCT/US 99/27341

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROSENBERG, A.: "Purification of human.beta.- glucosyl ceramidase from cultured cells for potential enzyme replacement therapy in Gaucher's disease" PROC. FEBS MEET. (1980), 61(IND. CLIN. ENZYMOL.), 77-87 , XP000915137 the whole document	28-36
A	WO 98 26662 A (SCHENGRUND CARA LYNNE ;PENN STATE RES FOUND (US); THOMPSON JEFFREY) 25 June 1998 (1998-06-25) page 1, line 8 - line 11 page 4, line 5 -page 7, line 17 page 9, line 27 -page 10, line 4 page 13, line 20 -page 14, line 7 claims 1-3,7,10-15,17,18,20	49-54
A	EP 0 352 766 A (FIDIA SPA) 31 January 1990 (1990-01-31) the whole document	55-63, 72-74
P,X	PURI A ET AL: "The neutral glycosphingolipid globotriaosylceramide promotes fusion mediated by a CD4-dependent CXCR4-utilizing HIV type 1 envelope glycoprotein" PROC NATL ACAD SCI USA, vol. 95, 24 November 1998 (1998-11-24), pages 14435-14440, XP002141785 cited in the application the whole document	1-10, 12-19
P,X	US 5 962 302 A (CORLEY NEIL C ET AL) 5 October 1999 (1999-10-05) column 1, line 10 - line 55 column 2, line 57 - line 60 column 4, line 60 -column 5, line 34 column 18, line 19 -column 19, line 15 column 21, line 1 -column 22, line 28	20-27
P,X	US 5 955 293 A (ACHESON DAVID W K ET AL) 21 September 1999 (1999-09-21)	72
P,A	the whole document, especially column 8, line 33 - column 9 line 21	55-63, 73,74

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-74, 79-84

   globotriaosylceramide used by preference of glycosphingolipids, different uses thereof and use of various inhibitors/blocking agents either directed against globotriaosylceramide or for synthesis inhibition

2. Claims: 75-78

   screening assay for cell fusion regulators using CD4-cells and gp120 comprising cell (or virus or liposome)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 99/27341

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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